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PROCEEDINGS OF THE BIOCHEMICAL SOCIETY

The **281st Meeting** *of the Biochemical Society was held at the London School of Hygiene and Tropical Medicine, Keppel Street, London, W.C.* 1, *on Saturday,* 12 *November* 1949, *and took the form of a Symposium on 'The Biological Oxidation of Aromatic Rings'.*

COMMUNICATIONS

The Oxidation of Aromatic Rings by Purely Chemical Means. By REGINA SCHOENTAL. (*Department of Chemistry, University of Glasgow*)

Oxidation of aromatic rings in homolytic and heterolytic reactions was discussed, including dehydrogenation, oxidation by substitution, by addition, and subsequent more drastic one, resulting in ring fission.

The last three types of oxidation are known to occur in living organisms (e.g. phenol and muconic acid are formed from benzene; dihydroxy-dihydro-derivatives from naphthalene and tricyclic aromatic hydrocarbons), therefore special attention was given to the methods, which by purely chemical means yield products similar in type (though mainly isomeric) to the metabolites formed from the parent compounds *in vivo*. A review was given of the products of the oxidation of benzene and its homologues, naphthalene, and several other polycyclic aromatic hydrocarbons, obtained by the action of organic peracids, peroxides, and hydrogen peroxide, in the presence of catalysts, such as oxides of Os, V, Fe, Se, etc., that is, of elements having the common feature of being able to change reversibly their several oxidation levels.

Examples were given illustrating the relation between the reactivities assigned to different positions of aromatic polycyclic hydrocarbons on wave-mechanical grounds, and the type of oxidizing agents by which the respective positions are attacked.

A novel type of pure chemical oxidation of benzene was described, and its bearing on the interpretation of the metabolic fate of this hydrocarbon in living organisms considered.

The Orientation of Biochemically Introduced Hydroxyl Groups in Benzene Derivatives in the Animal Body. By J. N. SMITH. (*Department of Biochemistry, St Mary's Hospital Medical School, London, W.* 2)

A survey of the positions taken up by the OH groups when aromatic compounds are oxidized in the body is of value in predicting the fate of new compounds and of elucidating the mechanism of the enzymic reaction.

When occurring alone in the benzene ring, normally *o*-, *p*-directing groups direct the biochemically introduced OH group to the expected position. Supposed species differences in the *o*, *p* ratio in aniline-acetanilide metabolism are considered to be fortuitous and associated with the ease of deacetylation in different species.

Hydroxylation of benzenes monosubstituted with normally *m*-directing groups is only recorded in the cases of nitrobenzene and benzoic acid which give rise to *p*-phenols and cyanobenzene which is hydroxylated *o*, *m*, and *p* to the —CN group.

While the results with the *o*-, *p*-directing groups can be interpreted in terms of a cationic mechanism and with the *m*-directing groups by an anionic process, it is unlikely that both can occur together. A more reasonable interpretation is afforded by the assumption of a free radical hydroxylation process, in which the radical (possibly not a OH radical) is transferred without being truly free. The reaction may thus be a transhydroxylation.

Application of the principles to disubstituted benzenes must take account of the slower reaction *o* and *p* to a $+E$ substituent.

The Oxidation of Polycyclic Hydrocarbons in the Animal Body. By L. YOUNG. (*Department of Biochemistry, St Thomas's Hospital Medical School, London, S.E.* 1)

Although polycyclic hydrocarbons undergo certain metabolic changes analogous to those which occur with benzene (e.g. naphthalene and anthracene, like benzene, are converted to mercapturic acids), it is not known whether this is generally true of the metabolic changes to which these compounds are subjected. Benzene undergoes ring fission in the animal body with the formation of muconic acid, but with the exception of acenaphthene, little is known about the occurrence of ring fission in the metabolism of polycyclic hydrocarbons. Oxidation of side chains occurs during the metabolism of alkylbenzenes, but it is not known whether similar changes take place in the metabolism of alkyl-substituted polycyclic hydrocarbons such as methylcholanthrene.

It is generally accepted that oxidation of benzene in the organism leads to the formation of phenols which are excreted in the urine in the free form and in conjugation with sulphuric acid and glucuronic acid. Free and conjugated phenols have also been isolated from excreta following the administration of polycyclic hydrocarbons (e.g. naphthalene, chrysene, fluorene, 1:2:5:6-dibenzanthracene, 3:4-benzpyrene). It has been shown, however, that some polycyclic hydrocarbons are metabolized to dihydroxy-dihydro derivatives which are excreted in the urine (e.g. naphthalene is converted to 1:2-dihydronaphthalene-1:2-diol). These diols can be decomposed in acid solution to yield phenols, and while it seems unlikely that all the phenols isolated from the excreta of animals dosed with cyclic hydrocarbons are artefacts, the position will remain uncertain until more is known about the stability of the diols. If it is established that the phenols isolated from excreta following the administration of cyclic hydrocarbons are not artefacts, it will be important to determine whether diols are intermediates in the metabolic formation of these phenols.

The Biological Significance of Metabolism of Polycyclic Hydrocarbons. By E. BOYLAND. (*The Chester Beatty Research Institute, London, S.W.* 3)

Aromatic hydrocarbons and related compounds produce specific biological effects which are difficult to relate to their metabolic processes. The leucopenic action of benzene and the effect of naphthalene in inducing cataract in rabbits must be related to one or other of the metabolic changes which these substances undergo. In so far as the chemical combination of a drug with a body constituent is a metabolic change any pharmacological action brought about by any means other than physical effects must involve metabolism of the agent. Some metabolic processes may be detoxification processes, while others are essential for the biological effect produced.

Several hundred aromatic carcinogens are known, including polycyclic hydrocarbons and nitrogenous compounds which produce certain specific biological effects. These include (1) induction of cancer in susceptible animals, (2) inhibition of growth of normal and malignant cells, (3) induction of mutations in animals and (4) production of specific damage to chromosomes. These effects, which are radiomimetic, can also be induced by physical agents which obviously do not involve metabolism of the agents although the agents cause changes in metabolism in the cells effected.

Most of the carcinogenic compounds with the exception of urethane have chemically reactive groups. The French theoretical chemists (cf. Daudel & Pullmann, 1945) drew attention to the 'K region' (which possesses appreciable double bond character) present in carcinogenic hydrocarbons. This double bond reacts with osmic acid or perbenzoic acid. The dimethylaminophenyl group also reacts with perbenzoic acid and is a grouping frequently found in carcinogenic compounds. Carcinogens may require (among many properties) two reactive groups which may be double bonds, tertiary amine groups or, as in the nitrogen mustards, active chloroethyl groups.

Investigation of metabolism of carcinogenic hydrocarbons has shown that the oxidation does not occur at the active double bond or even at the meso positions in the case of anthracene derivatives, but that the elements of hydrogen peroxide are added at positions other than those of primary chemical reactivity. This suggests that the compounds are combined to some tissue constituent through the K region by an addition reaction (cf. Boyland, 1948). The saturation of the double bond by this process would cause other bonds in the molecule to be activated, and such bonds may then be oxidized. The real carcinogenic complex may be the complex between the tissue constituent and hydrocarbon or the oxidation product of this complex. The oxidative metabolism may be a detoxification process, but some evidence suggests that the metabolism may be associated with carcinogenesis.

The fact that the dihydroxy-dihydro derivatives which are found in hydrocarbon metabolism are *trans* derivatives (Booth & Boyland, 1949) suggests

that they may arise by hydrolysis of an epoxide formed by union of the hydrocarbon with oxygen. Such an epoxide might be hydrolysed enzymatically or combine with a tissue group such as a phosphoric ester. Another alternative is that the oxidation is a process coupled with phosphorylation. The primary combination of hydrocarbons through the K region or double bond might involve a preliminary formation of an epoxide but it is simpler to consider the double bond reacting with some tissue constituent directly until more evidence on this point is available.

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The Biological Oxidation of Alicyclic Amino-acids in Mammals. By ROSALIND PITT-RIVERS. (*National Institute for Medical Research, Hampstead, N.W.* 3)

Phenylalanine, an essential amino-acid for mammalian growth, is the parent substance of many biologically important compounds. The introduction of one oxygen atom into the benzene ring leads in nature to only one compound, tyrosine. Further ring oxidation of tyrosine (or its derivatives) can lead to two groups of compounds: (i) those which undergo *ortho* oxidation, i.e. 3:4-dihydroxyphenylalanine, melanin and adrenaline, and (ii) those which undergo *para* oxidation, i.e. homogentisic acid and thyroxine; homogentisic acid is generally regarded as a normal intermediate in tyrosine metabolism; further oxidation results in ring fission and the formation of acetoacetic acid.

Certain metabolic disorders may result in only partial oxidation of phenylalanine derivatives. Failure to oxidize the benzene nucleus at all is found in phenylpyruvic oligophrenia; here, only oxidative deamination of the side-chain is achieved. Tyrosinosis and avitaminosis C both lead to the excretion of *p*-hydroxyphenylpyruvic and lactic acids; here *para* oxidation of tyrosine is interfered with, and breakdown of the ring is prevented. In alcaptonuria, the failure of normal tyrosine metabolism occurs at a later stage, perhaps at that of ring fission itself; the excretion of homogentisic acid by alcaptonurics shows that neither *para* oxidation of the ring nor deamination of the side-chain have been prevented.

The various mechanisms proposed to explain these different reactions and the enzyme systems responsible for some of them were described and discussed.

Biological Aromatization of Hydroaromatic Compounds. By FRANK DICKENS. (*Courtauld Institute of Biochemistry, The Middlesex Hospital Medical School, London, W.*1)

The pathways of biological oxidation of aromatic and hydroaromatic compounds differ so widely that their comparison is of value in defining those features which are special to the aromatic ring. In addition, so little is known of the biological precursors of aromatic or partially aromatic substances in the animal body that it may be profitable to indicate by what routes and mechanisms this transformation is already known to occur. This is particularly desirable since much speculation has centred round more complex aspects of the problem such as the possibility of the origin of sex hormones and carcinogenic compounds from sterols or bile acids (cf. Cook, 1943), about which no definite opinion can yet be expressed.

Among the simpler aromatization reactions the work of Bernhard (1937, 1945) in Zurich has established the ability of the intact dog, man or rabbit to convert *cyclo*hexane carboxylic acid and some of its derivatives to benzoic acid or its analogues. By the use of deuterium the increased urinary excretion of benzoic (or hippuric) acid was shown to originate from the hydroaromatic acid which was fed or injected. Dickens (1947) showed that isolated liver (or, to a less extent, kidney) slices of the rabbit could also aromatize *cyclo*hexane carboxylic acid, and the hippuric acid formation was confirmed by its isolation. This aerobic reaction requires intact liver slices and even fortified homogenates which vigorously oxidize fatty acids such as octanoic acid (Schneider, 1948) are found to have lost completely the ability to aromatize this ring system (Beer, Dickens & Pearson, 1949*a*, *b*).

The mechanism of this reaction has been studied by the use of possible intermediates in the aromatization. Whereas none of the seven monohydroxy

*cyclo*hexane benzoic acids was aromatized in the rabbit, all three tetrahydrobenzoic acids were converted into benzoic acid, suggesting a dehydrogenative rather than a hydroxylative mechanism. Among other sources of benzoic acid in the animal body, quinic acid is of interest as a postulated dietary source of part of the benzoic acid normally excreted in human urine.

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The Oxidation of Aromatic Rings by Micro-organisms in Metabolism. By F. C. HAPPOLD.
(*Department of Biochemistry, University of Leeds*)

Historical introduction. The oxidative breakdown of aromatic hydrocarbons. The integration of knowledge resulting from studies on bacterial oxidases and tyrosinase with that acquired in the field of the microbiological metabolism of cyclic amino-acids, aromatic carboxylic acids and phenols. Some comparisons between the mammalian and microbiological metabolism of some of these compounds were given and some attention was paid to the experimental methods used.

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PROCEEDINGS OF THE BIOCHEMICAL SOCIETY

The **282nd Meeting** *of the Biochemical Society was held in the Department of Biochemistry and Chemistry, Guy's Hospital Medical School, London, S.E.*1, *on Friday,* 16 *December* 1949, *when the following papers were read:*

COMMUNICATIONS

A Method for the Rapid Estimation of Salicylates in Serum or Plasma. By M. J. H. Smith and J. M. Talbot. (*Department of Chemical Pathology, King's College Hospital Medical School, Denmark Hill, London, S.E.* 5)

Photometric methods for the determination of salicylate levels in blood employ the colours given with ferric nitrate (Brodie, Udenfriend & Coburn, 1944), with nitric acid and sodium hydroxide (Volterra & Jacobs, 1947), with copper sulphate and sodium nitrate (Mallick & Rehmann, 1945) and with an alcoholic solution of the Folin-Ciocalteu phenol reagent and sodium carbonate (Weichselbaum & Shapiro, 1945). These methods are relatively time-consuming, and the first three require 1 ml. or more of plasma, a quantity which is not always available, especially from children.

The procedure to be described requires only 0·2 ml. of plasma or serum and can be completed within 15 min. It is based on the reaction of salicylates with an aqueous solution of the Folin-Ciocalteu phenol reagent. A blue colour having an absorption maximum at 670 mμ. develops on the addition of sodium hydroxide. The absorption density of the coloured solution was found to be directly proportional to the concentration of salicylate ion up to 75 mg./100 ml., and the method gave a recovery of 97 % from plasma or serum. A calibration curve may be constructed from dilutions of salicylates in distilled water.

Seventy blood samples from patients not receiving salicylate therapy showed a range of blank values from 4·5 to 9·5 mg./100 ml., the arithmetical mean being 7·5 mg./100 ml. Substances which react with the reagent to give a blue colour and which may contribute towards the blank value include tyrosine, tryptophan and uric acid. It has been found that 2 mg. of uric acid are equivalent in colour production to 1 mg. of salicylate ion.

The method of estimation is as follows: 0·2 ml. of serum or plasma is added to 5·8 ml. of distilled water. 3 ml. of Folin-Ciocalteu reagent (diluted 1 to 3 with distilled water) are added and the mixture centrifuged for 5 min. 4 ml. of the supernatant liquid are removed and 2 ml. of 1·5 N-sodium hydroxide added. The solution is allowed to stand for 3 min. and the absorption density is measured against distilled water in a photoelectric absorptiometer using a 1 cm. cell and an Ilford spectrum red filter no. 608 transmitting maximally above 670 mμ.

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A New Naturally Occurring Amino-acid. By Elizabeth Work. (*Department of Chemical Pathology, University College Hospital Medical School, London, W.C.* 1)

An unknown ninhydrin-reacting substance (spot 17) was shown by paper chromatography to be present in acid hydrolysates of the water and ethanol-insoluble portion of *Corynebacterium diphtheriae* (Work, 1949). The substance has now been isolated in crystalline form and identified as $\alpha\epsilon$-diaminopimelic acid,

$$COOH.CH(NH_2).(CH_2)_3.CH(NH_2).COOH,$$

(synthesized by Sørensen, 1908).

The isolation of this new amino-acid was made possible by using paper chromatography for following the compound through each stage in the process. The neutralized acid hydrolysate was run through a large column of acid-treated alumina (Wieland, 1942) which retained the acidic amino-acids and, with sufficient alumina, the unknown amino-acid as well. The retained amino-acids were eluted with ammonia and the concentrated eluate was subjected to electrodialysis. The unknown

amino-acid was electrolytically neutral and remained in the centre compartment; it was thus separated from the acidic amino-acids which migrated to the anode. The contents of the centre compartment yielded on concentration a white crystalline precipitate which proved to be the unknown amino-acid.

Recrystallization produced needle-shaped crystals which analysed as $C_7H_{14}N_2O_4$ (Found: C, 44·3; H, 7·45; N, 14·8 %. Calc. C, 44·2; H, 7·42; N, 14·8 %); the nitrogen was present entirely as α-amino N, as shown by the Van Slyke ninhydrin CO_2 method. Benzoylation produced a compound (m.p. 181–199°) which after several recrystallizations (needles) melted at 193°. A crude sample of synthetic dibenzoyl-αε-diaminopimelic acid, kindly provided by Prof. Linderstrøm-Lang, melted over the same range, and after recrystallization melted at 195–197° (mixed melting point with natural benzoyl derivative 193–195°). Analytical figures for the natural benzoyl derivative were: C, 63·7; H, 5·88; N, 6·89 % (dibenzoyl-diaminopimelic acid, $C_{21}H_{22}N_2O_6$, requires C, 63·3; H, 5·57; N, 7·04 %). The differences in melting point and crystalline form of the natural and synthetic compounds could be attributable to the presence of two optically active C atoms in diaminopimelic acid. Pimelic acid was isolated as a degradation product after treatment of the natural amino acid with nitrosyl bromide and catalytic reduction of the resulting dibromo compound.

Natural and synthetic diaminopimelic acids behaved identically on phenol-collidine chromatograms.

This new amino-acid is present chiefly in the insoluble portion of *C. diphtheriae*, where it constitutes 4–8 % of the total protein (expressed as amino acid N × 100/total N). A small amount is also found in the ethanolic extract of the cells. Spots corresponding exactly with what was then my unknown amino acid were also found some time ago by Dr Synge in chromatograms from bacteria of sheep's rumen and by Asselineau, Choucroun & Lederer in an antigenic product of human T.B. These workers have kindly kept me informed of their progress. I subsequently found the spot in chromatograms from four strains of T.B. cells, human, avian, B.C.G. and bovine; also from *B. proteus*, but not from *Staph. aureus*.

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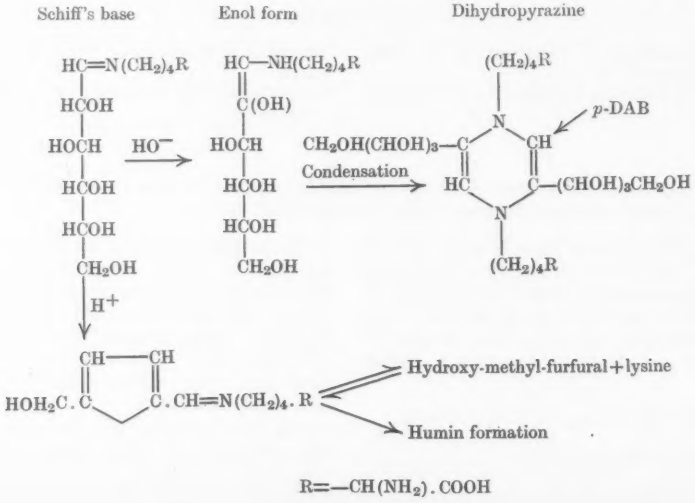
The Interaction Between Simple Sugars and Amino-acids. By A. GOTTSCHALK (*Walter and Eliza Hall Institute of Medical Research, Melbourne, Australia*) and S. M. PARTRIDGE (*Low Temperature Research Station, University of Cambridge*)

It was recently observed that a sugar derivative containing nitrogen (obtained enzymatically from ovomucin) decomposed with humin formation instantaneously upon mild acid hydrolysis, whereas alkali treatment and addition of *p*-dimethylamino-benzaldehyde (*p*-DAB) resulted in the production



of a purple colour (Gottschalk & Lind, 1949). Vasseur (1949) reported that a mixture of galactose with certain amino-acids or with methylamine gave rise to a purple colour when treated with *p*-ABD under suitable conditions.

The following results were obtained when a mixture of L-lysine and D-glucose adjusted to pH 8·5 with NaOH was allowed to interact at 37° and at a relative humidity of 70 % for 5 days.

(*a*) Examination by paper chromatography (Partridge, 1948, 1949) of the brown-coloured material revealed besides free lysine and glucose the presence of a compound reacting strongly with ninhydrin, weakly with aniline phthalate; this compound had a R_F value lower than both L-lysine and D-glucose in the solvents used.

(*b*) The compound referred to, after removal of the excess free glucose and upon treatment with alkali, reacted with *p*-DAB to give a stable purple colour. A similar colour was obtained when benzylamine D-glucoside was treated in the same way.

(*c*) On treatment with 2N-oxalic acid at 100° the compound readily formed 5-hydroxymethyl furfural (HMF), besides soluble and insoluble humin substances.

Furthermore, it could be shown that L-lysine, when heated with HMF in glycerol, was deaminated in the α-position to an extent of about 95 % (cf. Akabori, 1933). To account for the observations made, the following scheme is suggested.

The existence of part of the lysine D-glucoside in the open Schiff's base form (double bond) accounts for the ease of its conversion to HMF and humin by mild acid treatment. This interpretation is borne out by the finding that aniline D-glucoside forms HMF and humin under acid conditions so mild as not to affect free D-glucose, D-fructose or D-arabinose.

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The Measurement of Vitamin B_{12} by Biological Assay with Chicks. By M. E. Coates, G. F. Harrison and S. K. Kon. (*Cod-Liver Oil (Poultry) Standardization Laboratory, National Institute for Research in Dairying, University of Reading and the Agricultural Research Council*)

Chicks have been used by many American workers (e.g. Nichol, Robblee, Cravens & Elvehjem, 1949; Stokstad, Jukes, Pierce, Page & Franklin, 1949; Lillie, Denton & Bird, 1949) for the biological estimation of vitamin B_{12}, but none of the methods so far suggested has been subjected to close statistical analysis. Our design of the assay allows assessment of its accuracy.

The diet used throughout was similar to that of Rubin, Bird & Rothchild (1946) and had the following percentage composition: ground maize 38·6, ground barley 20, defatted soya grits 35, dried grass 3, bone meal 1·5, limestone flour 1, NaCl 0·672, $MnSO_4.4H_2O$ 0·028, arachis oil 0·2. To each 100 g. diet were added: vitamin A 400 i.u., vitamin D_3 50 B.S.I. units, riboflavin 0·15 mg., nicotinic acid 1 mg. Standard and unknown were incorporated into the diet.

Since normal chicks carried reserves of vitamin B_{12}, American workers found it necessary to breed chicks from hens on all-vegetable diets. It seemed possible that chicks commercially bred in this country might not have such reserves, but our tests showed that, as purchased, they were unsuitable for the assay. Removal of yolk sacs from day-old commercial chicks did not materially reduce the reserves, and an attempt to deplete them more rapidly by addition to the chicks' diet of 0·05 % iodinated casein to increase their metabolic rate, met with little success.

Newly hatched chicks were therefore obtained from hens depleted of vitamin B_{12} by the method described in the accompanying communication (Black, Getty, Coates, Harrison & Kon, 1949). They showed signs of deficiency of vitamin B_{12} manifested by a weight of only about 160 g. at 4 weeks; this was significantly improved by administration of a purified liver extract (Examen, Glaxo Laboratories Ltd.) containing vitamin B_{12}, treated chicks reaching a weight of about 250 g. at 4 weeks.

Such depleted chicks responded equally to microbiologically equivalent doses of vitamin B_{12} contained in crystalline vitamin B_{12}, in Examen and in crude-liver extract. Examen was therefore adopted as our standard source of vitamin B_{12}.

With this substance a linear relationship was found at dose levels of vitamin B_{12} of 1–3 μg./100 g. diet between body weight at 4 weeks and the

logarithm of the dose; with groups of ten chicks and three dose levels the slope (b) was 67·8 with an error of 35·2.

The test proved applicable to the assay of natural products although the limits of error were large, and for two samples of fish solubles, kindly supplied by Dr J. Lovern, of the Torry Research Station, Aberdeen, the results shown in the table were obtained. They are much higher than those obtained microbiologically by Mr G. E. Shaw, Evans Biological Institute, Runcorn, with *Lactobacillus lactis* (Dorner) or by Mr F. W. Wilby of this Laboratory, also microbiologically, with *Euglena gracilis*. We are investigating the causes of this discrepancy.

We are greatly indebted to Dr T. F. Macrae and his colleagues of Glaxo Laboratories Ltd. for the crystalline vitamin B_{12} and the liver preparations of known vitamin B_{12} potency.

Sample	No. of birds in group	No. of groups: Standard	No. of groups: Unknown	Vitamin B_{12} in μg./g.: Value found	Vitamin B_{12} in μg./g.: True fiducial limits at $P=0·95$
1	6	3	2	1·27	0·36–2·28 (28–179 %)
2	6	3	3	0·30	0·16–0·46 (54–155 %)

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Hatchability of Eggs and the Production of Chicks for the Assay of Vitamin B_{12}. By D. J. G. Black and J. Getty (*Department of Agriculture, University of Reading*) and M. E. Coates, G. F. Harrison and S. K. Kon (*Cod-Liver Oil (Poultry) Standardization Laboratory, National Institute for Research in Dairying, University of Reading, and the Agricultural Research Council*)

It was shown in the previous communication (Coates, Harrison & Kon, 1949) that specially prepared chicks are needed for the assay of vitamin B_{12}. For the production of such chicks hens were kept on an all-vegetable diet of the following percentage composition: ground maize 22·5, ground wheat 20, ground oats 20, defatted soya grits 25, dried grass 5, bone meal 4·2, limestone 2·3, salt mixture* 0·5, cod-liver oil 0·5; about 5 mg. riboflavin were added to each pound of diet.

Two groups of twelve Light Sussex hens, each group with a Rhode Island Red cock, were kept in folds on raised wire screens to prevent access to vitamin B_{12} in the faeces. After the hens had been a month on this diet, eggs were collected in fortnightly batches and incubated in the usual way. The first chicks hatched from them had lower reserves of vitamin B_{12} than normal chicks, as shown by their growth on the all-vegetable chick diet of Coates *et al.* (1949), and these reserves became gradually less in successive hatches. At the same time hatchability decreased from about 80 to about 30 % in 6 months. The hens themselves remained in good condition, and post-mortem examination of four of them revealed no gross pathological changes, but histological study of certain organs is in progress. A similar effect on hatchability on vegetable rations has been reported by several workers (Lindstrom, Petersen, Wiese & Moore, 1949; Lampman, Petersen & Bolin, 1944; Bird, Rubin, Whitson & Haynes, 1946). After 8 months the diet of one group of hens was supplemented with a vitamin B_{12} concentrate (Examen, Glaxo Laboratories Ltd.), supplying about 13 μg. vitamin B_{12}/lb. of food. The hatchability of eggs from these hens improved immediately, but for the group not given the supplement it remained very low. The vitamin B_{12} reserves of the chicks from the hens given the supplement gradually increased until their growth on the all-vegetable protein diet was little different from normal.

* $MnSO_4.4H_2O$, 6 g.; KI, 0·06 g.; NaCl to make 100 g.

Our best thanks are due to Dr T. F. Macrae and to his colleagues of Glaxo Laboratories Ltd for the preparation of Examen of known vitamin B_{12} potency.

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The Distribution of Cholinesterase Types in Autonomically Innervated Tissues. By M. G. Ord and R. H. S. Thompson. (*Department of Chemical Pathology, Guy's Hospital Medical School, London, S.E.* 1)

In extension of the work of Sawyer & Everett (1947) on the distribution of cholinesterase types in different tissues, a comparison has been made of the ability of homogenates of various rat tissues to hydrolyse acetylcholine (ACh), benzoylcholine (BCh) and acetyl-β-methylcholine (MCh).

In contrast to nervous tissue and skeletal muscle, it has been found that smooth muscle (small intestine), cardiac muscle (ventricle and auricle), and certain glandular tissues (salivary gland, Harderian gland and intestinal mucosa) hydrolyse BCh either at the same rate as MCh or significantly faster. Summation experiments with BCh and MCh have indicated that different enzymes are concerned with the hydrolysis of these substrates in these tissues. Determinations of the concentrations (I_{50}) of di-*iso*propyl fluorophosphonate (DFP) required to cause 50 % inhibition of hydrolysis of ACh, BCh and MCh have shown that the enzyme hydrolysing BCh in these sites is very much more sensitive to inhibition by this compound than that hydrolysing MCh. The evidence obtained suggests therefore that the esterases which we have studied in these tissues correspond to the 'true' and 'pseudo' cholinesterases described by Mendel & Rudney (1943) in brain, red blood cells and serum. Suprarenal gland, liver, lung and skin have also been studied, and the results obtained with them have supported the conclusion that, of the rat tissues which we have investigated, those which receive only post-ganglionic autonomic innervation exhibit an activity towards BCh either equalling or exceeding that towards MCh, thus contrasting with rat brain and skeletal muscle which, as was known from earlier work, exhibit only slight activity towards BCh.

This work is now being extended to human tissues, and an active pseudo-cholinesterase has been demonstrated in certain areas of brain and in uterus, ureter and jejunal muscle and mucosa. In placenta, perfused with tap water until the effluent fluid was no longer visibly blood-stained, only a very low order of activity towards BCh has been found, whereas the hydrolysis of MCh by this tissue proceeds rapidly.

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Supplementary Enzyme in Brown Adipose Tissue. By N. H. Creasey and C. H. Gray. (*Department of Chemical Pathology, King's College Hospital, Denmark Hill, London, S.E.* 5)

Cori & Cori (1943) found that in the presence of extracts of liver and heart, crystalline muscle phosphorylase (*a*) catalysed the synthesis from glucose-1-phosphate of a branched polysaccharide similar to glycogen. In the absence of such extracts a linear polysaccharide similar to amylose was formed. They attributed this activity of the extracts to the presence of a supplementary enzyme which catalysed the introduction of the branch points into the polysaccharide molecule.

The present investigation is concerned with the possible occurrence of the supplementary enzyme in the brown adipose tissue of rats. It is known that large quantities of glycogen are deposited in this tissue when carbohydrate is fed after a period of starvation (Tuerkischer & Wertheimer, 1942).

The isoelectric precipitate at pH 5·85 was removed from an aqueous extract of rat brown adipose tissue and the supernatant stirred with C_γ alumina; after centrifuging, active material was eluted from the alumina with 0·25 M-sodium β-glycerophosphate and the eluate was adjusted to 40 % saturation with ammonium sulphate. The ammonium sulphate precipitate was dissolved in distilled water, dialysed and used in the tests for supplementary enzyme activity.

When the purified extract was incubated with glucose-1-phosphate, crystalline muscle phosphorylase (*a*) and 0·005 % of glycogen to prime the reaction the liberation of inorganic phosphate followed an autocatalytic curve and the polysaccharide formed stained red with iodine. Samples of the reaction mixture were taken before and after an hour's incubation, boiled to destroy all enzyme activity, and their ability to prime polysaccharide synthesis compared by adding them to a system containing only crystalline muscle phosphorylase (*a*) and glucose-1-phosphate. The reaction rate curves showed that the combined action of crystalline muscle phosphorylase (*a*) and adipose extract had brought about an increase in the concentration of branched polysaccharide as shown by a definite increase in priming ability.

10 mg. of the polysaccharide synthesized by the

combined action of the crystalline phosphorylase (*a*) and brown adipose tissue extracted were isolated. It stained red with iodine, was soluble in water and had about half the priming activity of an equal concentration of glycogen when tested with crystalline muscle phosphorylase (*a*). It is concluded that brown adipose tissue contains supplementary enzyme.

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Comparative Studies of 'Bile Salts'. 1. Preliminary Survey. By G. A. D. Haslewood and Veryan Wootton. (*Guy's Hospital Medical School, London, S.E.* 1)

Bile occurs in all vertebrates, and the 'bile salts' are perhaps to be regarded as physiologically its most important constituent. It is therefore of considerable interest that the chemical nature of the 'bile salts' is by no means invariable from species to species, but rather appears to show some evidence of evolutionary development.

We have examined for the first time the bile of species of Teleostii (1), Amphibia (1), Reptilia (12), Mammalia (19); we have also repeated the examination of several other biles and of a pig's gallstone. We have isolated the following substances: from the frog, *Rana temporaria*, a new ?pentahydric alcohol, as sulphate; from the Nile monitor, *Varanus niloticus, varanic acid*, $?C_{27}H_{46}O_8$; from the Boidae (3 species), *pythonic acid*, $C_{24}H_{40}O_5$. We have also good evidence for the existence of other new bile acids in reptilian bile. The pig's gallstone yielded a derivative of β-hyodeoxycholic acid ($3\beta:6\alpha$-dihydroxycholanic acid).

Present knowledge about the distribution of 'bile salts' may be partially summarized as follows: Elasmobranchii (6 species), scymnol sulphate, ?cholic acid, $?C_{27}$ acids; Teleostii (30 species), cholic acid, $?C_{27}$ acids; Amphibia (3 species), $?C_{27}$ alcohols as sulphates, C_{27} acids; Reptilia: (*a*) Lacertilia (1 species), varanic acid, (*b*) Serpentes (15 species), cholic, pythonic and ? unknown acids; (*c*) Chelonia (3 species), $?C_{27}$ acids; Aves (6 species), chenodeoxycholic, cholic acids; Mammalia, all species, except pig and nutria rat, cholic, deoxycholic and chenodeoxycholic acids in varying proportions: in seals and walruses, also phococholic acid. We have confirmed the existence of nutriacholic acid, but we have not encountered ursodeoxycholic acid in the bile of three species of bears.

The possible relationship of the nature of the chief constituents of 'bile salts' to the position of the species in the evolutionary scale will be discussed.

We owe acknowledgements (to be made in detail later) to many who have helped us with bile collections.

Comparative Studies of Bile Salts. 2. The Nature of Pythonic Acid. By G. A. D. Haslewood and Veryan Wootton. (*Guy's Hospital Medical School, London, S.E.* 1)

Pythonic acid, m.p. 166–167°, occurs in the bile of the Western Boa, *Constrictor occidentalis*, the Indian Python, *Python molurus*, and the African Python, *Python sebae*; in the last species, it appeared to be the main constituent. The formula of crystalline pythonic acid, by elementary analysis and titration, is $C_{24}H_{40}O_5$, it readily gave *pythonic lactone*, $C_{24}H_{38}O_4$, m.p. *c.* 270°.

Chromic oxidation of pythonic lactone gave *bis dehydropythonic lactone*, $C_{24}H_{34}O_4$, H_2O, m.p. 137–139°, which with NaOH gave an acid, which was methylated with diazomethane to yield (partially purified) *methyl bis dehydropythonate*, $C_{25}H_{38}O_5$, m.p. 143–144°. Chromic oxidation of this substance gave a ketone, *methyl ter dehydropythonate*, probably $C_{25}H_{36}O_5$, m.p. 127–130°, and this on Kishner-Wolff reduction gave what appeared to be cholanic acid, m.p. 158–159°, not depressed by an authentic sample. (Found: C, 80·1; H, 11·1%. Calc. for $C_{24}H_{40}O_2$: C, 80·0; H, 11·1%.) These experiments suggest that the molecule of pythonic acid has the cholanic acid skeleton and that it contains three secondary hydroxyl groups, including one which takes part in lactonization. One of the other groups may be at C_{12}, as expected from the difficulty of complete acetylation. On biogenetic grounds the third group may be put at C_3. Hence, pythonic acid may possibly be a 3:12:15 or 16-trihydroxycholanic acid.

The Solubility of Enamel Nitrogen. By D. J. Anderson (introduced by G. A. D. Haslewood). (*Physiological Laboratory, Guy's Hospital Medical School, London, S.E.* 1)

In the early stages of its formation, dental enamel shows an organic matrix, which cannot be demonstrated in the fully formed tooth by ordinary histological techniques depending on decalcification. This may be explained by mechanical destruction during bubble formation, or solution of the organic material in the decalcifying fluid. In order to test the second possibility, and at the same time obtain information concerning the nature of the enamel nitrogen, determinations were made of nitrogen partition after dissolving enamel in various acids. Preliminary results show that enamel nitrogen has a marked solubility in the acids so far tested.

Organic Constituents of Dentine. By M. V. Stack. (*Department of Dental Medicine, Guy's Hospital, London, S.E.* 1)

Five fractions were obtained by applying a method used to estimate bone collagen (Lightfoot & Coolidge, 1948). Crushed vacuum-dried dentine from normal teeth was washed to remove 3–6 % of the total N. Tannic acid precipitated two-thirds of this water-soluble N. After autoclaving with water for 1 hr. at 130°, the washed dentine no longer contained collagen, judging by the cessation of N extraction. This extract, containing 80–85 % of the total N (3·4 %), was freed from gelatin by tannic acid. In analyses on five pairs of recently erupted premolars, the collagen was found to account for 75 ± 2 % of the total N, the tannic acid filtrate containing another 7 %. The residue was immediately soluble in 0·5 N-HCl, and nearly all the N (11–15 %) was precipitable with tannic acid. A similar nitrogen partition was observed in a few samples of cortical bone.

Bone and dentine powders acceptable for protein analysis should be obtained by crushing in a percussion mortar. The same amount of time devoted to filing or grinding procedures results in a destruction of collagen equivalent to that observed when crushed dentine was heated for 2 hr. at 150°. However, the amounts of acid-soluble protein N and non-protein N remained the same under all conditions of preparation.

The present figure for collagen N (2·6 %) indicates 14 % of this protein in dentine; a further 5 % may be accounted for by the remaining N (0·8 %). An estimate of the polysaccharide, shown by about 3·5 % reducing substances (Rogers, 1949), may be added to the 0·8 % citric acid reported (Free, 1943), and to the 0·2 % ether-soluble material. This sum amounts to four-fifths of the 28 % organic matter (Bird, Gallup, Gaudino & Hodge, 1948).

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DEMONSTRATIONS

Estimation of Trichloroethylene in Blood. By F. H. Brain and P. Helliwell (introduced by G. A. D. Haslewood). (*Guy's Hospital Medical School, London, S.E.* 1)

Comparative Studies of Bile Salts. By Veryan Wootton. (*Guy's Hospital Medical School, London, S.E.* 1)

A **Three-Dimensional Scatter Diagram.** By J. N. Hunt (introduced by G. A. D. Haslewood). (*Guy's Hospital Medical School, London, S.E.* 1)

The Estimation of Nitrogen in Dental Enamel. By D. J. Anderson (introduced by G. A. D. Haslewood). (*Physiological Laboratory, Guy's Hospital Medical School, London, S.E.* 1)

(*a*) The preparation of uncontaminated enamel samples.
(*b*) Micro-digestion.
(*c*) Ammonia determination in the Conway micro-diffusion unit. A modified rocking apparatus was shown.

A Case of Virilizing Tumour of the Ovary. By W. H. H. Merivale. (*Department of Clinical Pathology, Guy's Hospital, London, S.E.* 1)

FORTHCOMING PAPERS

It is hoped to publish the following papers in the next issue of the *Biochemical Journal*:

A micro method for separating free and esterified vitamin A. By E. Eden

Hydrolysis and esterification of vitamin A during absorption. By E. Eden and K. C. Sellers

The metabolism of sulphapyridine containing radioactive sulphur in the rabbit. By H. G. Bray, G. E. Francis, F. C. Neale and W. V. Thorpe

The fate of certain organic acids and amides in the rabbit. 10. The application of paper chromatography to metabolic studies of hydroxybenzoic acids and amides. By H. G. Bray, W. V. Thorpe and K. White

Metabolism of derivatives of toluene. 4. Cresols. By H. G. Bray, W. V. Thorpe and K. White

Further observations on changes in β-glucuronidase activity in the mouse. By Lynda M. H. Kerr, J. G. Campbell and G. A. Levvy

Urinary excretion of pregnanediol in human subjects following the administration of progesterone and of pregnane-3α:20α-diol. 1 and 2. By I. F. Sommerville and G. F. Marrian

Some properties of acridane and 2-chloro-7-methoxyacridane: their possible relationship to excretion products of mepacrine. By A. L. Tárnoky

The effect of oxygen and of cocarboxylase on the formation of citrate and α-ketoglutarate by pigeon brain homogenates. By R. V. Coxon and R. A. Peters

The reactivity of the iminazole ring in proteins. By R. R. Porter

Studies on the synthesis of lactose by the mammary gland. 1. Precursors. By F. H. Malpress and A. B. Morrison

The valences of protein ions from electrophoretic and membrane potential measurements. By P. A. Charlwood

The selective hydrolysis of amylopectin and amylose by β-amylase. By R. H. Hopkins and B. K. Jha

On the mechanism of secretion of ions by gastric mucosa and by other tissues. By R. E. Davies and A. G. Ogston

Displacement chromatography on synthetic ion-exchange resins. 5. Separation of the basic amino-acids. By S. M. Partridge, R. C. Brimley and K. W. Pepper

Water-miscible solvents in the separation of amino-acids by paper chromatography. By H. R. Bentley and J. K. Whitehead

The distribution of cholinesterase types in mammalian tissues. By M. G. Ord and R. H. S. Thompson

The virulence-enhancing factor of mucin. 1. A biological assay of virulence-enhancing activity. By H. Smith

On the state of hyaluronic acid in synovial fluid. By A. G. Ogston and J. E. Stanier

[*The Editors of the Biochemical Journal accept no responsibility for the Reports of the Proceedings of the Society. Abstracts of papers read are published as received from the author.*]

PROCEEDINGS OF THE BIOCHEMICAL SOCIETY

The **283rd Meeting** *of the Biochemical Society was held at the British Postgraduate Medical School, Ducane Road, Hammersmith, London, W.* 12, *on Saturday,* 21 *January* 1950, *when the following papers were read:*

COMMUNICATIONS

Observations on the Chemistry of Hyaluronic Acid. By M. A. G. Kaye and M. Stacey. (*Department of Chemistry, University of Birmingham*)

A modified technique has been devised for the isolation from human umbilical cord of hyaluronidin, a mucoprotein containing hyaluronic acid, and for isolating the latter, protein-free. D-Glucuronic acid, acetic acid and D-glucosamine, as components of hyaluronic acid, have been identified by isolation of derivatives and by the application of paper-strip chromatography.

Use of the methylation techniques, well-tried in polysaccharide chemistry, has failed so far in this case to give clear-cut results.

It appeared that in the relatively undegraded acid-polysaccharide macromolecule, only a relatively low content of hydroxyl groups were free to form methyl ether residues.

Degradation of the hyaluronic acid could be achieved under acid or alkali conditions to give a mixture of products some of which, on subsequent methylation, yielded methylated derivatives in the form of syrups, having relatively high methoxyl contents. Acid hydrolysis of these gave some products which were clearly monosaccharide derivatives but in the main the split products, particularly those fragments containing nitrogen, were oligosaccharide derivatives remarkably resistant to further acid hydrolysis. The only product which it has been possible to define was a dimethyl ether of methyl glucopyruronoside methyl ester, which, on further methylation, gave rise to fully methylated glucopyruronic acid (identified as the crystalline amide). It is possible to state that in the degraded material only very few, if any, of the glucuronic acid residues can be present as terminal groups and in these uronic acid units some of the carboxyl groups remain free and some are bound in either ester or amide formation or in some other way.

The methylated oligosaccharide units contain a relatively high proportion of nitrogen and appear to be closely related to chitin-like derivatives which form a remarkably stable 'core' in hyaluronic acid. From paper-strip chromatography it appeared also that nitrogen-containing units of a non-carbohydrate nature were present in hyaluronic acid. It is clear that techniques and methods new to carbohydrate chemistry will need to be developed in order to unravel the complex structure of hyaluronic acid.

It seems reasonably certain that the prevailing idea of hyaluronic acid as a polysaccharide acid containing alternating units of equal amounts of *N*-acetyl glucosamine and glucuronic acid is untenable. It is possible that the *N*-acetyl amino sugar units form a chitin-like 'core' to which is attached a complicated polyglucuronic acid structure and a prosthetic group.

Attempts are now being made to separate and identify labile residues by cautiously graded hydrolysis and to identify products obtained by the acetolysis technique.

Observations on the Structure of the Specific Polysaccharide of *Azotobacter chroöcoccum*. By G. J. Lawson and M. Stacey. (*Department of Chemistry, University of Birmingham*)

The viscous acid polysaccharide synthesized by *Azotobacter chroöcoccum* (Cooper, Daker & Stacey, 1938) reacts in high dilution with Types III and VI pneumococcus antiserum (M. Heidelberger, private communication). This same behaviour is exhibited by the capsular polysaccharide of another nitrogen-fixing organism, namely *Rhizobium radicicolum* (clover strain) which was shown to be related structurally to the Type III pneumococcus polysaccharide inasmuch as it possessed a cellobiuronic acid type of unit (Schlüchterer & Stacey, 1945). The structure of the *Azotobacter* polysaccharide has now been examined by classical and chromatographic methods. Aqueous hydrolysis followed by paper chromatography revealed the presence of glucose, galactose and glucuronic acid residues only. Methylation with dimethyl sulphate and sodium hydroxide afforded an almost colourless homogeneous methyl

ether (OMe, 39 %) soluble in a mixture of chloroform and acetone giving highly viscous solutions. This ether was broken down in a stepwise manner by graded hydrolysis with methanolic HCl, yielding mixtures of methylhexosides and an oligosaccharide acid separated as a barium salt. The mixture of hexosides could not be satisfactorily separated by fractional distillation in a high vacuum owing to the formation of azeotropes, though it was possible to show the complete absence of fully methylated hexose 'end groups'.

The partially methylated hexoses in the azeotropes could not satisfactorily be converted into anilides, but the presence of D-glucose and D-galactose was confirmed by conversion to their crystalline tetramethyl derivatives.

A chromatographic examination (kindly carried out by Dr J. K. N. Jones) of the main part of hexoside fractions, on columns of cellulose (Hough, Jones & Wadman, 1949), showed them to consist of 2:3:6-trimethylglucose, together with smaller amounts of 2:4:6-trimethylglucose, 2:4:6-trimethylgalactose, with an as yet unidentified dimethylhexose(s). A similar examination of the glucuronic acid containing oligosaccharide showed it to be a trisaccharide type composed of 2:4:6-trimethylglucose, a dimethyl (possibly 2:3) glucose, and a partially methylated glucuronic acid.

The data, given with some reserve, indicated a possible repeating unit for the polysaccharide composed of three D-glucose units and one D-galactose molecule and one D-glucuronic acid unit. The D-galactose is linked through positions 1 and 3, while the glucose is linked partly through positions 1 and 4 and partly through positions 1 and 3. The polysaccharide clearly possesses a branched chain structure with the carboxyl groups of the uronic acid residues free to engage in salt formation, such a structure closely resembling that of the plant gums.

Similarities to the Type III pneumococcus polysaccharide structure (Reeves & Goebel, 1941) are shown in the possession of the glucose-glucuronic acid type of aldobiuronic acid residues and in the fact that both polysaccharides contain a significant proportion of 1:3-glycosidic linkage. The relationship to the *Rhizobium* polysaccharide lies in the aldobiuronic acid residues.

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Microbiological Assay of Pure Vitamin D_2 and Vitamin D_3; Possible Function of Non-ionic and Ionic Lipids for the Bacterial Cell. By E. Kodicek. (*Dunn Nutritional Laboratory, University of Cambridge and Medical Research Council*)

The assay procedure has been based on previous findings of the bacteriostatic effect on *L. casei* of *cis*-forms of long-chain unsaturated fatty acids (Kodicek & Worden, 1944, 1945, 1946). The bacteriostasis could be reversed by several substances of which vitamins D_2 and D_3 were the most potent. The reversing effect of vitamin D was proportional to its concentration, within a range of 0·5–4 μg./ml. of medium. The technique required strict standardization of the inoculum and addition of an amount of linoleic acid (*c.* 20 μM) just sufficient to effect complete cessation of growth and acid production. Vitamin D_3 gave quantitatively the same response. The variation between experiments was considerable, nevertheless, it was no greater than the variation observed in the initial stages of other microbiological vitamin assays. Tests for specificity on seventy different substances showed that of the fat-soluble compounds, only cholesterol, vitamin E, lecithin, and to a lesser degree coprostanone, interfered (Table 1); work is in progress to ascertain whether the assay can be made specific for vitamin D. Promising results were obtained in preliminary trials with the non-saponifiable matter of some

Table 1. *Reversal effect by fat-soluble substances of linoleic acid inhibition*

Active	Inactive
Vitamin D_2	7-Dehydrocholesterol
Vitamin D_3	Lumisterol Dihydrocholesterol
Cholesterol	Cholestanone Ergosterol Zymosterol Sitosterol Coprostanone (±) Testosterone Desoxycorticosterone acetate
Lecithin	Lecithin, saponified
α-Tocopherol	α-Tocopherol, saponified(±) Vitamin A alcohol Vitamin A acetate

[Ca^{++}]

natural products containing vitamin D; fish liver oils, however, contained a potent inhibitor which interfered with the assay.

Apart from the practical application, the theoretical aspect is of interest. The bacteriostatic effect of linoleic acid can be explained primarily on a physico-chemical basis (barrier theory; Kodicek, 1948, 1949) which does not exclude the possibility that unsaturated fatty acids have also a metabolic function. By the adsorption and penetration of linoleic acid into the lipoprotein patches of the cell wall of the bacteria, the surface pressure is increased and may distort spatially and functionally the protein lattice. Such substances as vitamin D reverse this effect. Small amounts of vitamin D (*c.* 10–20 μM) in absence of linoleic acid were toxic, and so were high amounts of the vitamin even in the presence of linoleic acid. The growth-promoting properties of vitamin D and of calcium salts and the toxic action of the former are reminiscent of similar findings in higher organisms.

The author wishes to thank Prof. Friedemann and Mr A. L. Bacharach for their precious gift of various sterols and Mr D. R. Ashby for his valuable technical assistance.

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The Metabolism of Halogenobenzenes. Isolation of a Dihydrodihydroxychlorobenzene and other Metabolites from Chlorobenzene Urine. By B. SPENCER and R. T. WILLIAMS. (*Department of Biochemistry, St Mary's Hospital Medical School, London, W.* 2)

In the past, studies on the metabolism of the halogenobenzenes have been mainly concerned with their ability to form mercapturic acids. These acids, however, only account for about 20–25% of the halogenobenzenes fed. The present work shows that the main bulk of these compounds are oxidized in the rabbit and twice as much of the halogenobenzenes are excreted as O-conjugates as in the form of S-conjugates. The chlorophenols form no mercapturic acids and give only O-conjugates (see Table 1).

Chlorobenzene urine has been examined in detail and some of the metabolites isolated and identified as follows:

(*a*) *Diol of chlorobenzene.* Chlorobenzene urine was adjusted to pH 7·5 and continuously extracted with ether for 16 hr. Acidic constituents were removed from the extracts with alkali; from the dried ether extract *dihydroxydihydrochlorobenzene* (m.p. 129–130° and $[\alpha]_D - 413°$ in water) was isolated. (Found: C, 49·1; H, 4·7; Cl, 24·1%; $C_6H_7O_2Cl$ requires C, 49·1; H, 4·8; Cl, 24·2%.) The yield was minute, being 20·5 mg. from 90 g. of chlorobenzene fed. It was shown spectroscopically to be a $\Delta^{1:3}$-hexadiene compound which was completely dehydrated by 0·1N-HCl in 35 min. at 98° to *p*-chlorophenol. This suggests that it is *l*-3:4-dihydroxy-3:4-dihydrochlorobenzene.

(*b*) p-*Chlorophenol and* 4-*chlorocatechol.* These were isolated in small yields by direct ether extraction of the urine at pH 5. The two phenols were separated by lead acetate precipitation and identified as *p*-toluene-sulphonates. The same phenols were isolated in good yields after mild acid hydrolysis of the urine; the yields of 4-chlorocatechol and *p*-chlorophenol were in the ratio of 20 to 1.

Table 1

	Percentage of dose excreted as		
	O-conjugates†		S-conjugates†
Compound*	Ethereal sulphates	Glucuronides	Mercapturic acid
Chlorobenzene	27	25	20
Bromobenzene	37	40	21
Iodobenzene	30	31	23
o-Chlorophenol	19	82	0
m-Chlorophenol	21	68	0
p-Chlorophenol	13	88	0

* Doses in molecular equivalents of 150 mg. chlorobenzene/kg.
† Assuming that monoconjugates are formed.

(*c*) p-*Chlorophenylglucuronide.* This substance wa obtained in small yield from the glucuronide gum and characterized as its *amide* (m.p. 245–246° decomp.; $[\alpha]_D - 85·6°$ in methanol). This amide was also prepared biosynthetically by feeding *p*-chlorophenol to rabbits.

(*d*) *Glucuronide of* 4-*chlorocatechol.* This substance was isolated in large yield after gentle acid hydrolysis of the urine or of the glucuronide gum. It was characterized as 4-chloro-2-methoxyphenylglucuronidamide and was identical with the glucuronide

obtained from the urine of rabbits fed with 4-chlorocatechol (Dodgson & Williams, 1949). Our experiments suggest that this compound is an artefact but the nature of its precursor has not yet been established.

(*e*) p-*Chlorophenylmercapturic acid.* This was isolated during prolonged ether extraction of chlorobenzene urine and had m.p. 153° and $[\alpha]_D - 20°$ in ethanol.

The above results were discussed.

REFERENCE

Dodgson, K. S. & Williams, R. T. (1949). *Biochem. J.* **45**, 381.

The Interactions of Purified Serum Albumins with Calcium. By N. H. MARTIN and D. J. PERKINS.
(*Department of Chemical Pathology, St George's Hospital, London, S.W.* 1)

MacLean & Hastings (1935) first attempted a quantitative study of the relationship of calcium to horse and bovine albumins prepared by fractionation of the original sera with ammonium sulphate and further purification by electrodialysis. Further studies were carried out by Weir & Hastings (1936) and by Drinker, Green & Hastings (1939). In the last ten years there have been considerable advances in the techniques of protein fractionation and purification. It seemed useful, therefore, to re-investigate the problem with the products now available.

In the present investigation albumins of varying purities from three different species, horse, ox and human, were examined. Though the purity of each specimen was assessed by ultra-centrifugal and electrophoretic analysis, it was appreciated that the processes by which separation had been achieved might result in modifications of the intimate structure of the albumin molecule not appreciable by either of these physico-chemical techniques. For this reason albumins separated by a variety of techniques from each species were studied. In all five different samples of human albumin, one of horse, and three of bovine, were examined.

Preliminary investigations showed that, under the standard conditions chosen, equilibrium was achieved in four days. Seven days were allowed in each of the groups of experiments reported. The pH of the system was measured when equilibrium had been achieved, the Donnan effect was determined experimentally and a correction applied, and, in every case, interactions were studied over a range of protein concentrations.

It will be seen that the combining power of horse and ox albumins are of the same order, and are substantially greater than that of the human albumins purified and examined under comparable conditions.

Table 1

Albumin	Separation	Approximate purity electrophoretic analysis (%)	Ca mg. / Protein N g. (mean of readings)	Range of proteins conc. studied (g./100 ml.)	pH range studied
1. Horse	Crystallized. Kekwick. Carbohydrate-poor fraction*	100	11·8	0·7–2·8	6·3–6·5
2. Ox (*a*)	Pillemer alcohol fractionation	94–97	11·2	1·4	6·8
3. Ox (*b*)	Armour preparation	90–95	4·1	6·0	6·9
4. Ox (*c*)	Crystallized from alcohol fractionation†	100	2·2	2·8–5·6	6·1–6·3
5. Human (*a*)	Pillemer alcohol fractionation	94–97	4·7	1·4–3·0	6·6–6·7
6. Human (*b*)	Ether fractionation of Kekwick and MacKay*	85	4·8	2·0–4·0	6·6–6·7
7. Human (*c*)	25% albumin (American Red Cross) for therapy	95–97	Nil	6·0	5·8–8·4
8. Human (*d*)	Five times recrystallized alcohol fractionation†	100	4·7	2·5–5·0	6·7–6·85
9. Human (*e*)	Decanol recrystallization from alcohol fractionation†	100	1·1	1·8–5·0	6·2–6·4

* We are indebted to Dr Kekwick for these specimens.
† We are indebted to the Department of Physical Chemistry, Harvard, for these specimens.

It demonstrates further that repeated crystallization may alter the capacity of the albumin to bind calcium, either by modification of the molecule or by removal of a specific fraction responsible for calcium binding, and that techniques of purification vary in their effect as measured by calcium binding.

Because stabilizers might have been used in the preparation of albumin No. 7 (Boyer, Lum, Ballou, Luck & Rice, 1946), the effect of the presence of acetyl tryptophane on the binding capacity of other albumins was investigated but no interference could be demonstrated.

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A Biochemical Description of Locust Coloration. By T. W. Goodwin and S. Srisukh. (*Department of Biochemistry, The University, Liverpool* 3)

Immature adult (male and female) Desert Locusts (*Schistocerca gregaria*) are predominantly pink, whilst mature males are yellow and mature females are brown. The two major pigments in these locusts are β-carotene (Goodwin & Srisukh, 1949; Goodwin, 1949) and acridioxanthin; the latter being a redox pigment of unknown constitution first mentioned by Chauvin (1941). In the immature and mature adults of both sexes there is very little variation in the amounts of these pigments present, so qualitative differences must be sought to explain the colour changes. It has been found that the pink of the immature insects is due to the presence of free reduced acridioxanthin, the brown of the mature females to reduced acridioxanthin attached to a tissue constituent, in all probability a protein, and the yellow of mature males to β-carotene which is revealed, in the body segments at least, partly by the withdrawal of the acriodioxanthin to the edges of the segments and probably also partly by the translocation of the β-carotene from the subcutaneous tissues into the hypodermis.

When the complex colour patterns of the integument of the pink and brown insects are considered it is found that the patterns are produced merely by the differential accumulation of acridioxanthin over the surface of the integument; even the very dark narrow markings of the legs are due to the same pigment. 'Melanin' is apparently confined to the wings, mandibles and leg joints. Similar considerations can be applied to the Migratory Locust (*Locusta migratoria migratorioides*).

The brown patches found on some solitary locusts are also produced by acridioxanthin, but the frequently occurring green patches contain a complex (very similar to Junge's (1941) 'insectoverdin') composed of orange-yellow astaxanthin and β-carotene chromoproteins together with a blue chromoprotein with probably glaucobilin as its prosthetic group, and a yellow water-soluble substance.

Coloured plates were produced to illustrate these findings.

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Utilization of a Casein Supplement Administered to Ewes by Ruminal and Duodenal Fistulae. By D. P. Cuthbertson and Margaret I. Chalmers. (*Rowett Research Institute, Bucksburn, Aberdeenshire*)

When casein is fed or administered into the rumen of a sheep there is an immediate rise in the ammonia concentration of the rumen which reaches a peak in 2 hr. and then steadily declines (McDonald, 1948*a*). A rapid rise occurs in the ammonia concentration of the venous blood draining the rumen (McDonald, 1948*b*) after the administration of casein into the rumen (McDonald, 1948*a*).

It was found that pregnant ewes on different levels of nutrition excrete 60–70 % of the nitrogen supplied from a casein supplement of 50 g. per day by mouth. Nitrogen balance experiments on sheep receiving the casein supplement dripped through fistulae into the duodenum and rumen, show that the percentage retention of the casein-N when given by duodenal fistula is greater than that from casein dripped into

Table 1. *Nitrogen retained from a casein supplement of 50 g. per day*

Sheep		Basal N intake (g./day)	N intake + casein (g./day)	% N retained by (days of period in brackets) Duodenum	Rumen
N Low-plane*	Pregnant	6·06	12·21	49 (12)	18 (7)
O Low-plane	Pregnant	6·06	12·21	64 (7)	41 (12)
P Low-plane	Pregnant	6·06	12·21	50 (19)	—
Q Low-plane	Pregnant	6·06	12·21	—	34 (18)
R Low-plane†	Non-pregnant	9·20	15·66	18 (9)	Out>in§ (9)
S Low-plane	Non-pregnant	9·20	15·66	24 (9)	20 (9)
N Well-fed‡	Non-pregnant	24·90	31·40	Out>in (15)	6 (15)
					8 (15)
O Well-fed	Non-pregnant	24·90	31·40	17 (15)	—
				4 (15)	
				9 (15)	
P Well-fed	Non-pregnant	24·90	31·40	20 (15)	Out>in (15)
				11 (15)	
Q Well-fed	Non-pregnant	24·90	31·40	—	13 (15)
					Out>in (15)
					1 (15)

* Diet = meals 62½ g.; hay 250 g.; swedes 750 g. per day.
† Diet = meals 150 g.; hay 400 g. per day.
‡ Diet = meals 550 g.; hay 900 g. per day.
§ More N excreted than ingested from the casein supplement.

the rumen. The extent to which the nitrogen was retained also depended on the state of nutrition of the animal. Data are shown in Table 1.

It would appear that the rate of deamination of the casein in the rumen was in excess of the capacity to synthesise bacterial protein and therefore the ammonia formed was absorbed into the blood stream, converted to urea and excreted in the urine. Thus the low-plane pregnant ewes failed to use the casein supplement to meet their requirement for protein. In the well-fed animals the food consumed was sufficient to cover any loss due to deamination of protein in the rumen.

We wish to thank Dr A. T. Phillipson for carrying out the fistulae operations on the animals.

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Experiments on the Mechanism of the Formation of Methylthiol from *S*-methyl-L-Cysteine in Cultures of *Scopulariopsis brevicaulis*. By F. Challenger and A. G. Lowther. (*Department of Organic Chemistry, The University, Leeds*)

One of us and Charlton showed (1947) that in bread cultures of *Scopulariopsis brevicaulis* (Strain, Washington 2; N. Coll. T.C. No. 580) DL-methionine gives methylthiol and, by further methylation, dimethyl sulphide in (we find) a total yield of 15%. Similarly, *S*-alkyl-L-cysteines (alkyl = Me, Et and *n*-Pr) give alkylthiol and methylalkyl sulphide, in (we find) a total yield of 80%, with *S*-methyl-L-cysteine. Glucose-Czapek-Dox (G.-C.-D.) medium, adjusted to pH 7, has been used for a study of the mechanism of the *S*-methyl-cysteine fission by the same strain of *S. brevicaulis*. This may be (*a*) reductive, (*b*) hydrolytic or (*c*) may involve the elimination of CH_3S- and H- from adjoining carbon atoms, giving different products in each case. Alternatively, the α-keto acid $CH_3SCH_2COCOOH$ may be a primary product and then undergo (*a*) or (*b*).

The mould was grown in several flasks of the adjusted G.-C.-D. medium (200 ml.) containing 0·25% of *S*-methyl-L-cysteine. A flask was removed every 7–10 days, the contents filtered, concentrated, sterilized (autoclave or Seitz filter gave the same results) desalted and the medium examined by two-dimensional paper chromatography (phenol, collidine). In control experiments without *S*-methylcysteine, the cultures were similarly examined.

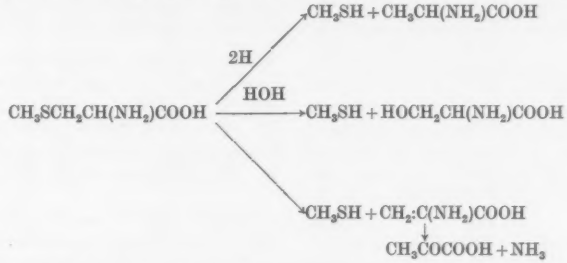
The first test-culture showed glycine, valine and leucine in fairly low concentration, aspartic acid and alanine in fairly large concentration and much *S*-methylcysteine. Later tests gave similar results, but the amount of amino-acids decreased and after 6 weeks aspartic acid only was found. No serine was ever detected. The control experiments revealed only a fairly high concentration of aspartic acid.

The electrolytic desalting apparatus was shown to produce no alanine from *S*-methyl-L-cysteine.

The identification of alanine was confirmed by addition of authentic alanine to the solution to be examined, when the concentration, as shown by the intensity of the ninhydrin colour reaction, was increased and the two spots were exactly superimposed.

four days. The pyruvic acid was recovered as its 2:4-dinitrophenylhydrazone. It appears reasonable to conclude that the disappearance of the alanine is due to its conversion to protein.

These preliminary results have no quantitative significance, but the absence of serine and of pyruvic acid and the relative intensity of the alanine 'spot'



The disappearance of the alanine (also observed on addition of alanine to G.-C.-D. cultures of the mould) may be due to utilization in protein formation or to oxidative deamination to pyruvic acid. This was not detected by alkali titration or addition of 2:4-dinitrophenylhydrazine. Pyruvic acid added to cultures on G.-C.-.D medium did not disappear (tests as before) during 21 days in agreement with the results of Barnard (1947), who found no fall in alkali titre and no formation of acetaldehyde during

suggest a reductive fission of methylcysteine. It is hoped, following a suggestion by Dr C. E. Dent, to examine the behaviour of *S*-methyl-DL-cysteine with the mould. As D-alanine would presumably not be utilized in protein synthesis it—or possibly pyruvic acid arising by deamination—might be expected to accumulate.

We gratefully acknowledge the help of Dr Dent who allowed one of us to work in his laboratory, and also the interest of Dr W. S. Reith.

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The Seasonal and Species Distribution of Oestrogen in British Pasture Plants. By S. P. Legg (*The National Institute for Research in Dairying, Shinfield*), D. H. Curnow and S. A. Simpson (*The Courtauld Institute of Biochemistry, Middlesex Hospital, London, W.* 1)

A review of the literature (Löve & Löve, 1945) points out that the occurrence of oestrogens in plants was first observed by Loewe & Spohr (1926), and that the animal sex hormones have since been demonstrated in plant material ranging from the bacteria to the flowering plants. The role of these substances in plants has not been fully explained, but that they may affect the reproductive system of the grazing animal and cause infertility has been demonstrated by Bennetts and his collaborators (Bennetts, 1944; Bennetts, Underwood & Shier, 1946). These workers devised a method of extraction and assay which made quantitative tests on plant material possible (Robinson, 1949). A preliminary examination which demonstrated the presence of oestrogens in English pasture has already been reported (Bartlett, Folley, Rowland, Curnow & Simpson, 1948). A more detailed investigation is in progress, and results obtained during 1949 are now reported. The method of extraction and assay has been essentially that of Robinson. The chemical nature of the oestrogen and the state in which it occurs in the plant are not yet known, and there is some doubt as to the efficiency of the extraction. Table 1 shows that considerable oestrogenic activity has been found both in grasses and clover. Lucerne (*Medicago sativa* L.), White Clover (*Trifolium repens* L.) and Timothy Grass (*Phleum pratense* L.), however, showed little activity. In each of the potent species, activity has been found in all parts of the plant tested, i.e. leaf, petiole, stem and inflorescence. There is considerable seasonal variation and the various plant organs do not show maximal concentrations simultaneously. It has also been found that when chloroplasts are precipitated from the juice obtained by crushing plant material, the bulk of the oestrogen occurs in the precipitate.

The results indicate that oestrogens occur in British pasture plants in concentrations which vary with the species. The early occurrence in the leaf and

Table 1. *Occurrence of oestrogens in Ryegrass, Cocksfoot and Broad Red Clover during the* 1949 *growing season*

(Oestrogenic activity is expressed as μg. oestradiol per 100 g. plant dry matter.)

Plant	Description of material	Date of sample during 1949: Feb. 1st half	Feb. 2nd half	Mar. 1st half	Mar. 2nd half	Apr. 1st half	Apr. 2nd half	May 1st half	May 2nd half	June 1st half	June 2nd half	Other samples
Perennial Ryegrass S 23 (*Lolium perenne* L.)	Leaf	—	—	—	—	4·5	—	4·5	0·2	0·2	—	0·3*
	Stem	—	—	—	—	3·2	—	5·0	0·8	Nil	—	
H.I. Ryegrass	Leaf	—	0·6	—	10·0	1·1	—	—	—	—	0·4	
	Stem	—	—	—	2·3	0·7	—	—	—	—	Nil	
Cocksfoot S 37 (*Dactylis glomerata* L.)	Leaf	—	—	—	1·5	2·4	—	1·4	0·4	Nil	0·3	Nil†
	Stem	—	—	—	Nil	Nil	—	0·1	2·3	Nil	—	
	Inflorescence	—	—	—	—	—	—	—	—	1·3	—	
Red Clover (*Trifolium pratense*)	Leaf	—	2·2	4·0	—	—	—	—	—	3·2	2·4	2·7‡
	Stem	—	—	1·9	—	—	—	—	—	0·9	0·9	

* 3 Oct. 1949. † 13 July and 3 Oct. 1949. ‡ 26 Nov. 1948.

the high concentration in chloroplasts suggest that this is the site of production. Various workers have shown that application of oestrogens stimulates vegetative growth (Bonner & Axtman, 1937; Orth, 1934; Zollikofer, 1936; see also Löve & Löve, 1945), but the findings listed above suggest that the high oestrogen concentration is associated with reproductive growth. Spring growth of grass, which is associated with high oestrogen concentration, precedes flowering but when autumn growth takes place the plant is not in a reproductive phase. Broad Red Clover, on the other hand, which has repeated high concentrations of oestrogen, will produce flowers throughout the summer and autumn.

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The Amino-Acid Pattern in Human Foetal and Maternal Plasma at Delivery. By H. R. Crumpler, C. E. Dent and O. Lindan. (*Medical Unit, University College Hospital, London, W.C.* 1)

Foetal and maternal blood collected from nine cases at the moment of birth has been analysed by the ninhydrin/CO_2 method (Hamilton & Van Slyke, 1943) for total α-amino-nitrogen (NH_2-N) and by paper chromatography (Consden, Gordon & Martin, 1944) for its content of individual amino-acids. The foetal plasma contained a higher total concentration of NH_2-N than the maternal.

The increase involved all the amino-acids commonly found in plasma. These results support the view that the placenta, by concentrating the amino-acid content of foetal blood, assists the foetus to synthesize proteins (Christensen & Streicher, 1948).

The lower foetal/maternal ratios (1·4 or less) for the plasma NH_2-N occurred in the four cases in which the mother had definite toxaemia of pregnancy. It is suggested that this may indicate that some impairment of placental function occurs in toxaemia.

	Foetal NH_2-N (mg./100 ml.)	Maternal NH_2-N (mg./100 ml.)	Ratio $\frac{\text{Foetal } NH_2\text{-N}}{\text{Maternal } NH_2\text{-N}}$
Mean	4·62	2·99	1·60
Range	2·94–6·70	2·23–3·70	1·03–3·00

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The Relationship of Acridanes to the Metabolism of Mepacrine in Man. By A. L. Tárnoky. (*Postgraduate Medical School, London, W.* 12 *and the Group Laboratory, Mile End Hospital, London, E.* 1)

The urine of subjects on mepacrine therapy contains substances which on spectrographic evidence may be diphenylamine or acridane derivatives (King, Gilchrist & Tárnoky, 1946). While the former are more stable, the latter should be formed more readily, and the deciding factor is the stability of acridanes in solution.

The rates of decomposition of acridane and 2-chloro-7-methoxyacridane at room temperature have been studied by an iodometric method. The results show the instability of these compounds which is most marked in acid solution. Thus when 0·01 M solutions in 90 % ethanolic 0·1 N-HCl are prepared, 71 % of acridane and 70 % of 2-chloro-7-methoxyacridane are lost in the first 24 hr. The instability of these compounds also interferes with the determination of their absorption spectra and acid solubilities. There are signs that the reaction is not always a simple oxidation to the corresponding acridine.

It is concluded that simple (5:10-unsubstituted) acridanes may be ruled out as the metabolic end-products of mepacrine as suggested by the spectral curves.

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The Nature of 'Inactive Haemoglobin'. By I. D. P. Wootton. (*Postgraduate Medical School, London, W.* 12)

Studies on 5-Nucleotidase and its Distribution in Human Tissues. By J. L. Reis. (Introduced by E. J. King.) (*Postgraduate Medical School, London, W.* 12)

The 5-nucleotidase is a phosphatase with a specific action: it acts only on the nucleotides with a phosphate group attached to the fifth carbon of the pentose, that is on the adenylic acid (adenosine-5-monophosphoric acid) and on the inosinic acid (inosine-5-monophosphoric acid). Its high degree of specificity was demonstrated in previous papers (Reis, 1937, 1939). It does not act on adenosine-triphosphoric acid, ribose-5-phosphoric acid, adenosine-3-monophosphoric acid or on any other phosphoric ester. The activity of the 5-nucleotidase is estimated by comparing the rates of hydrolysis of adenylic acid and phenylphosphoric acid. The latter is, as found by King & Delory (1939), a much more suitable substrate for the phosphatase than the β-glycerophosphoric acid, used previously for that purpose. Naturally the hydrolysis of the adenylic acid represents the sum of the 5-nucleotidase and phosphatase actions.

For comparison of 5-nucleotidase and phosphatase rabbits' lungs were used, as both enzymes are present in this tissue. As no method of separation of the 5-nucleotidase from the phosphatase could be found, for studying the 5-nucleotidase alone, a tissue must be chosen where that enzyme is abundant and the phosphatase present in traces only. Such conditions were found in man in the walls of the aorta and in the thyroid gland. The pH optimum of the 5-nucleotidase for those tissues was found between 7·5 and 8·0. An activation by the Mg ion could be demonstrated. The optimal substrate concentration is much lower for the 5-nucleotidase than for the phosphatase. Whereas according to Neumann (1949) the lowest optimal substrate concentration for the alkaline phosphatase is M/300, for the 5-nucleotidase the lowest value in some extracts is M/2400, but even at M/4800 the rate of hydrolysis is not appreciably smaller.

In human tissue the 5-nucleotidase is widely distributed. In bones, the typical site of the alkaline phosphatase, though the alkaline phosphatase at its optimal pH is several times more active than the 5-nucleotidase, at physiological pH's this condition is reversed, the adenylic acid being five times more

Table 1. *5-Nucleotidase and phosphatase activity of human tissues*

(μg. of phosphorus hydrolysed as phosphate, by an amount of extract corresponding to 20 mg. of tissue in 30 min. at 38°, at pH 7·5, substrate concentration M/1200.)

Tissue	Phenylphosphoric acid	Adenylic acid	Ratio: adenylic acid hydrolysis to phenylphosphoric acid hydrolysis
Ossifying cartilage	6	29	5
Kidney	7	17	2·5
Liver	1	9	9
Spleen	3	8	3
Lungs	3	18	6
Walls of aorta	0·5	34	68
Brain	0·5	15	30
Choroid plexus	3	24	8
Muscle (skeletal)	0·5	2	4
Pancreas	1	18	18
Thyroid	1	52	52
Testicle	1	38	38

easily hydrolysed than the phenylphosphoric acid. The 5-nucleotidase together with the phosphatase can be found also in lungs, kidney, brain and intestine. In nerves, walls of aorta, thyroid gland and in the testicles a 5-nucleotidase nearly free from phosphatase can be found (Table 1).

Taking into consideration that the phosphatase substrate in Robison's bone calcification theory (1932) is formed mainly by the adenylic acid (or by ATP) the importance of the 5-nucleotidase is obvious. It explains why calcification may proceed at physiological pH's in spite of the relative inactivity of the alkaline phosphatase in these conditions. Also in other tissues where calcification processes often take place (lungs, aorta, choroid) the presence of 5-nucleotidase can explain the chemical mechanism of the calcification on similar lines to that of the phosphatase in Robison's calcification theory.

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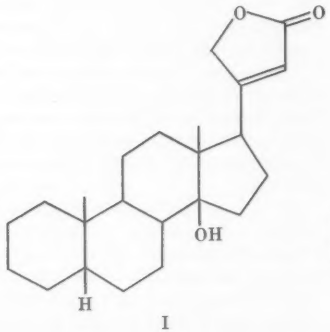
The Molecular Rotations of Cardiac Aglycones and Toad Poisons. By W. Klyne. (*Postgraduate Medical School, London, W.* 12)

The application of the method of molecular rotation differences to steroids containing many substituents is complicated by the fact that adjacent substituents may interfere with one another's rotation contributions ('vicinal action'; cf. Barton & Cox, 1948).

The rotation data for the cardiac aglycones and toad poisons (structures of Types I and II respectively) have now been analysed. The results show that, in general, substituents in ring D and in the side-chain do not interfere with the rotation contributions (Δ values) of substituents in the A and



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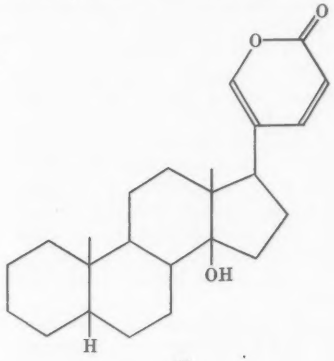

II

B rings (at C-3, C-5 and C-19). Some of these Δ values have been given by Barton & Klyne (1948); others are given in Table 1A. Conversely, substituents at C-3, C-5 and C-19 do not in general interfere with the Δ values of substituents in the D ring and side-chain (see Table 1B).

Since the Δ values given in Table 1 are approximately constant, they may be used in elucidating the structures of aglycones and toad poisons substituted only in rings A, B and D.

The few data available for compounds substituted at C-11 and C-12 show clearly that Δ values at these positions for 14-*n* compounds differ considerably from those for 14-*iso* compounds. Δ values for some C-ring substituents in 14-*iso* compounds may be independent of the nature of the side-chain.

Table 1. *Molecular rotation differences*

(Δ value = molecular rotation difference = $[M]_D$ of *X minus* $[M]_D$ of *Y*. Experimental error for Δ values is about ±10°. All data are for 5-*n* compounds, and are taken largely from recent papers by Reichstein, Meyer and their co-workers.)

Structure *X*	Structure *Y*	Δ value for *X*–*Y*: No. of examples	Range	Mean
A. Structural changes in A and B rings				
3(β):5(β)-$(OH)_2$	3(β)-OH	4	+41 to +51	+46
3(β)-OAc, 5(β)-OH	3(β)-OAc	5	+107 to +128	+120
3(β)-OAc, 5(β)-OH	3(β):5(β)-$(OH)_2$	5	+78 to +93	+87
3(β):5(β)-$(OH)_2$, 19-CHO	3(β):5(β)-$(OH)_2$	2	+54 to +58	+56
3(β)-OAc, 5(β)-OH, 19-CHO	3(β):5(β)-$(OH)_2$, 19-CHO	2	+80, +80	+80
B. Structural changes in D ring and side-chain				
14(β)-OH-14-*iso* aetio ester	14-*n*-aetio ester	3	−45 to −66	−53
Structure I	14(β)-OH-14-*iso* aetio ester	4	−33 to −56	−44
Structure II	Structure I	7	−96 to −113	−103

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Preparation, Titration Curves and Enzyme Hydrolysis of Carboxyphenylphosphates. By P. G. Walker and E. J. King. (*Postgraduate Medical School, London, W.* 12)

Some Observations on the Splanchnic Blood Flow in Man, Using the Hepatic Vein Catheterization Technique. By A. G. Bearn, Barbara H. Billing, J. C. S. Paterson and Sheila Sherlock. (Introduced by E. J. King). (*Postgraduate Medical School, London, W.* 12)

DEMONSTRATIONS

An Apparatus for the Continuous Extraction of Reducing Steroids from Urine. By J. P. Newhouse and W. Klyne. (*Postgraduate Medical School, London, W.* 12)

The acidified urine is extracted with chloroform in a liquid-liquid extractor, based on that of Robinson & Warren (1948) which was devised to avoid the persistent emulsions produced by hand-shaking in separating funnels. The extractor has been rendered automatic and self-adjusting by the use of a filter-pump, following the principle of the soil percolator of Lees (1948).

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King-Armstrong Phosphatase Estimation by the Determination of Liberated Phosphate. By E. J. KING, M. A. M. ABUL-FADL and P. G. WALKER. (*Postgraduate Medical School, London, W.* 12)

The phenylphosphate method of phosphatase estimation (King & Armstrong, 1934) utilizes the determination of enzymically hydrolysed phenol as a measure of the phosphatase activity of blood plasma and tissue extracts. For some purposes it is preferable to determine the liberated phosphate instead of the phenol, e.g. in the study of rickets in children where a knowledge of both the phosphatase and the inorganic phosphate of the plasma is useful, and in certain studies of the kinetics of phosphatase action. Since, in the hydrolysis of phenylphosphate, equimolecular amounts of phenol and phosphate are liberated, therefore, approximately 3 mg. of phenol (mol. wt. 94) are hydrolysed for each 1 mg. of inorganic phosphorus (at. wt. 31). The King-Armstrong unit of phosphatase is defined as the amount of enzyme which liberates 1 mg. of phenol in 30 min. at pH 9 and 37°, or in 15 min. at pH 10 and 37° (King, 1947). Using the same conditions of hydrolysis it is consequently necessary to multiply the number of mg. of liberated P by three, in order to obtain the same unitage of phosphatase. The following procedure gives close agreement with phosphatase determinations made by the estimation of phenol according to the phenylphosphate method.

Test: 3 ml. of 0·1 M-sodium carbonate-bicarbonate buffer pH 10 ($6Na_2CO_3$:$4NaHCO_3$) *plus* 3 ml. of 0·01 M-disodium phenylphosphate, warmed at 37° for 3 min.; 0·3 ml. plasma added, mixed and kept at 37° for 15 min.; removed from 37° bath and 1·2 ml. trichloroacetic acid (20 %) added to stop hydrolysis and precipitate proteins.

Control: 6 ml. water and 0·3 ml. plasma *plus* 1·2 ml. trichloroacetic acid.

Blank: 3 ml. buffer, 3 ml. phenylphosphate, 0·3 ml. water and 1·2 ml. trichloroacetic acid.

The mixtures are centrifuged or filtered, and 5 ml. filtrate (≡0·2 ml. plasma) treated with 0·8 ml. of 5 % ammonium molybdate in 15 % (v/v) H_2SO_4 and 0·2 ml. aminonaphtholsulphonic acid-reducing agent (0·2 of the 1:2:4-acid, 12 g. Na metabisulphite and 2·4 g. $Na_2SO_3.7H_2O$ in 100 ml. water).

Standard: 5 ml. phosphate standard (0·02 mg. P) *plus* 0·8 ml. ammonium molybdate and 0·2 ml. aminonaphtholsulphonic acid.

The colours are read in a photoelectric colorimeter with a red light filter.

Calculation:

Phosphatase units

$$= \frac{E \text{ of test} - (\text{control} + \text{blank})}{E \text{ of standard}} \times 0{\cdot}02 \times \frac{100}{0{\cdot}2} \times 3$$

$$= \frac{E \text{ of T} - (\text{C} + \text{B})}{E \text{ of standard}} \times 30.$$

Inorganic P (mg./100 ml.)

$$= \frac{E \text{ of control}}{E \text{ of standard}} \times 0{\cdot}02 \times \frac{100}{0{\cdot}2}.$$

An alternative micro procedure uses stannous chloride as the reducing agent for the phosphate estimations. *Test:* 1 ml. buffer, 1 ml. phenylphosphate, 0·1 ml. plasma, 15 min. at 37°, 7·9 ml. trichloroacetic acid (5 %). *Control:* 2 ml. water, 0·1 ml. plasma, 7·9 ml. trichloroacetic acid. *Blank:* 1 ml. buffer, 1 ml. phenylphosphate, 0·1 ml. water and 7·9 ml. trichloroacetic acid.

A *standard* (5 ml., containing 0·005 mg. P), and 5 ml. of each of the filtrates, are treated with 0·8 ml. of the 5 % molybdate in 15 % H_2SO_4 and 0·2 ml. of dilute stannous chloride (1 in 10 dilution, with 10 % H_2SO_4, of 10 % $SnCl_2$ in 5 N-HCl).

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An Improved Micro Acid-digestion Tube. By R. PARSONS and T. A. WEBSTER. (*Postgraduate Medical School, London. W.* 12)

Investigations on some Cellular Stain Reactions. By J. C. WHITE. (*Department of Pathology, Postgraduate Medical School, London, W.* 12)

Using Susa-fixed smears of biopsied human marrow and liver, intracellular distribution of nucleic acids has been studied with the aid of ribonuclease and desoxyribonuclease, staining by basic dyes, pyronin and methyl green, and by the Feulgen reaction.

Ribonucleic acid stains only with the pyronin. Nuclear desoxyribonucleic acid stains with both pyronin and methyl green, the relative affinity for the two dyes being influenced by the fixative, this factor possibly modifying the degree of polymeriza-

tion of the acid (see Kurnick, 1949), as well as affecting nucleoprotein linkages.

After removal of both nucleic acids by combined nuclease action or by 4% trichloroacetic acid extraction at 90° C. for 15 min. (Schneider, 1945; Pollister & Ris, 1947), the cellular proteins are still substantially intact, and stain with acid dyes below pH 4 in similar fashion to controls.

Using a mixture of orange G and aniline blue 707 at pH 2–3, globin of erythrocytes and, to a lesser extent, intranuclear histones retain orange G, whereas aniline blue more slowly stains plasma proteins and cytoplasmic proteins, including ribonucleoproteins. Some intranuclear structures, including chromonema threads, periphery of nucleoli and also the chromosomes finally stain deeply with aniline blue, possibly due to their content of non-histone proteins (Mirsky, 1947; Pollister & Ris, 1947) or of chromosomin (Stedman & Stedman, 1947). Similar staining of fixed films of proteins in the dye mixture shows that globin and histone* retain orange G strongly for a considerable period, whereas with ribonucleoprotein and desoxyribonucleoprotein complex, the initial orange G staining is rapidly replaced by aniline blue, as in the cytological preparations.

Extraction of ribonucleoprotein and desoxyribonucleoprotein complex from liver cells, by 0·85% and molar sodium chloride solutions respectively (Mirsky & Pollister, 1946), can be demonstrated cytologically by these staining methods, but with marrow cells extraction is more difficult.

In developing erythroblasts, the cytoplasmic affinity for orange G seems to follow the degree of formation of the globin moiety of haemoglobin, and it is noteworthy that the benzidine test for peroxydatic activity of haemoglobin becomes increasingly positive in these cells, in parallel fashion. The benzidine is dissolved in methanol containing 2·5% of acetic acid, and is followed by ozonic ether. Under these conditions, the peroxidase reaction of granular leucocytes is minimal.

* I am grateful to Prof. J. N. Davidson for a sample of histone, and to Drs E. M. and H. M. Jope for globin.

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Haldane Haemoglobin Determinations with the Photoelectric Colorimeter. By E. J. King and Maryse Geiser. (*Postgraduate Medical School, London, W.* 12)

The Haldane carbon monoxide haemoglobin standard defined by the British Standards Institute, and characterized by Donaldson, Harding & Wright (1943), furnishes a readily procurable and easily checked reference for the determination of haemoglobin in blood. The ordinary Haldane technique, in which a measured quantity of blood is saturated with CO and then diluted with water in a small test tube until its colour appears to match that of the standard, may give very inaccurate results (Macfarlane, 1945; Macfarlane, King, Wootton & Gilchrist, 1948). But the carbon monoxide procedure is nevertheless capable of yielding figures which conform to the true haemoglobin content of the blood with a high degree of accuracy, provided the assessment of the colour is made photometrically and with precision (King, Gilchrist, Wootton, Donaldson, Sisson, Macfarlane, Jope, O'Brien, Peterson & Strangeways, 1948). By employing photoelectric colorimeters (EEL, Gallenkamp, Hilger, etc.) and microadaptors which fit the B.S.I. Haldane standard and test tube, we have obtained results, much more easily and quickly than is possible by the dilution technique, which agree well with the most accurate colorimetric procedures and with iron and gas analyses.

A Haldane standard and test tube, marked only at the 2 ml. mark (100%), were obtained from Messrs Hawksley and Son, and the factor for conversion to true haemoglobin percentages of the ratio of test to standard readings was obtained by submission of the standard and test tube to the National Physical Laboratory, Teddington.

Blood (0·02 ml.) is washed from a capillary pipette into about 1 ml. of water (containing 0·4% of concentrated ammonia solution) in the test tube, and is saturated with carbon monoxide or with coal gas. Water (0·4% ammonia) is added carefully to the mark and the mixture gently shaken. The readings (extinctions) are then taken, of the test and the standard, in the photoelectric colorimeter fitted with a bright spectrum yellow-green light filter (Ilford 625) and the microadaptor for the Haldane tubes. The ratio of the readings (Test/Standard)

is multiplied by 100 and by the N.P.L. factor to give the concentration of haemoglobin in percentage of Haldane normal, or by 14·8 × the factor to give the haemoglobin in grams per 100 ml. of blood.

Results obtained by this procedure agreed with macro carbon monoxide determinations with a coefficient of variation of 2·1 %; with cyanhaematin determinations, 2·2 %; with oxyhaemoglobin by the M.R.C. photometer (King, Wootton, Donaldson, Sisson & Macfarlane, 1948), 2·3 %; and with iron analyses, 2·5 %.

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Increase in the Silicosis-producing Effects of Small Amounts of Quartz by Admixed Coal Dust. By E. J. King, S. C. Ray and C. V. Harrison. (*Postgraduate Medical School, London, W.* 12)

Determination of Iodine in Blood and Urine. By R. Fraser and Poppy Scarlet. (*Postgraduate Medical School, London, W.* 12)

[*The Editors of the Biochemical Journal accept no responsibility for the Reports of the Proceedings of the Society. Abstracts of papers read are published as received from the author.*]

PROCEEDINGS OF THE BIOCHEMICAL SOCIETY

The **Annual General Meeting** *of the Biochemical Society was held in the Department of Biochemistry, University College, Gower Street, London, W.C.* 1, *on Friday,* 24 *March,* 1950, *when the following papers were read:*

COMMUNICATIONS

Separation of *N*-2:4-Dinitrophenylamino-Acids by Partition Chromatography on Paper. By S. Blackburn and A. G. Lowther. (*Wool Industries Research Association, Torridon, Headingley, Leeds*)

N-2:4-Dinitrophenylamino-acids (DNP amino-acids) are of importance in determining the 'end-groups' of peptides and proteins, and can be separated on partition chromatograms on silica gel using water or other solvent as the stationary phase (Sanger, 1945). Effective separations of DNP amino-acids can also be effected on silica gel columns using phosphate buffers instead of water as the stationary phase, with solvents such as ether as the mobile phase (Blackburn, 1949).

In contrast to some large DNP peptides which appear to move satisfactorily on paper chromatograms, DNP amino-acids have generally given unsatisfactory results, due to the marked 'tailing' of the spots which is observed. Phillips & Stephen (1948) reported some success using a two-dimensional method, particularly with the slower moving DNP amino-acids, but also observed marked 'tailing' in many solvents.

We have now observed that DNP amino-acids may be successfully separated on one-dimensional paper partition chromatograms using paper previously soaked in phthalate or citrate buffers and dried in air, solvents used being saturated with buffer at room temperature. The DNP amino-acids then move down the paper as compact 'spots' with characteristic rates (R_F values), 'tailing' being eliminated.

A number of solvents have proved suitable. 1% butanol-chloroform is a suitable solvent for the separation of the faster moving DNP monoamino monocarboxylic acids, while ethyl acetate or *n*-butanol separate the slower moving DNP amino-acids from each other. The method has a high resolving power, a mixture of DNP-leucine, DNP-valine, DNP-alanine and DNP-glycine separating into four clearly defined spots in 30% propanol-cyclohexane. The possibility of performing end-group estimations on a micro scale is thus opened up.

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The Effect of *n*-Alkyl 3:5-Diiodo-4-hydroxybenzoates on Oxygen Consumption in Mice. By J. H. Wilkinson, M. M. Sheahan and N. F. Maclagan. (*Department of Chemical Pathology, Westminster Medical School, London, S.W.* 1)

The work of Frieden & Winzler (1949) on the effect of thyroxine analogues on metamorphosis in tadpoles has directed attention to the inhibitory properties of simpler compounds such as 3:5-diiodo-4-benzyloxybenzoic acid. Compounds of this type have also given results on oxygen consumption in mice suggesting active competition with thyroxine (Maclagan, Sheahan & Wilkinson, 1949). We have extended this work by the preparation of a series of new *n*-alkyl esters of 3:5-diiodo-4-hydroxybenzoic acid, which were tested for their effect on oxygen consumption in thyroxinized mice by a method described elsewhere (Maclagan & Sheahan, 1950). Inhibitory activity in this series has been demonstrated up to the *n*-octyl ester, the lower homologues, particularly the ethyl, propyl and butyl esters, being the most active. For example, the butyl ester produced a 62% inhibition of the thyroxine effect in a total dosage of 50 mg./kg. body weight (thyroxine dosage 2 mg./kg.).

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Utilization of Acetate for Milk-fat Synthesis in the Lactating Goat. By G. Popják, T. H. French and S. J. Folley. (*The National Institute for Medical Research, The Ridgeway, London, N.W. 7, and The National Institute for Research in Dairying, University of Reading*)

Synthesis of fatty acids and cholesterol has been shown to occur in the mammae of non-lactating pregnant rabbits (Popják & Beeckmans, 1950). It was also found that the short-chain volatile fatty acids obtained from the mammae of such rabbits are derived by synthesis from acetate and not by degradation of the long-chain fatty acids (Popják, Folley & French, 1949). The *in vivo* utilization of acetate for milk fat synthesis (particularly for the synthesis of volatile acids) confirmed the suggestion of Folley & French (1949*a*, *b*) based on their *in vitro* studies of lactating mammary tissues.

In the present experiment milk-fat formation was investigated with the aid of $CH_3{}^{14}CO_2Na$ in a lactating goat. The animal was injected intravenously with a single dose of 5 mc. of ^{14}C contained in 430 mg. of $CH_3{}^{14}CO_2Na$ dissolved in 0·9 % NaCl. Respiratory CO_2 was collected continuously for 6 hr. after the injection and the animal was milked at frequent intervals for 48 hr., after which time it was killed. The glyceride fatty acids extracted from the milk were separated into steam-volatile and non-volatile acids, the former having been further divided into water-soluble and insoluble fractions. The non-volatile acids were fractionated into saturated and unsaturated fatty acids by the lead salt method (Twitchell, 1921). The ^{14}C content of the fatty acids was then determined and is shown in Fig. 1. The specific activity-time curves of the milk fatty acids reached their maxima 3 hr. (volatile acids) and 4 hr. (non-volatile acids) after the injection and declined exponentially thereafter with a half-life of about 4 hr. Since the specific activity of the volatile fatty acids at their maxima was several times that of the long-chain non-volatile acids, it is quite clear that the volatile acids could not have originated from the degradation of the long-chain fatty acids but must have been synthesized from acetate or a derivative

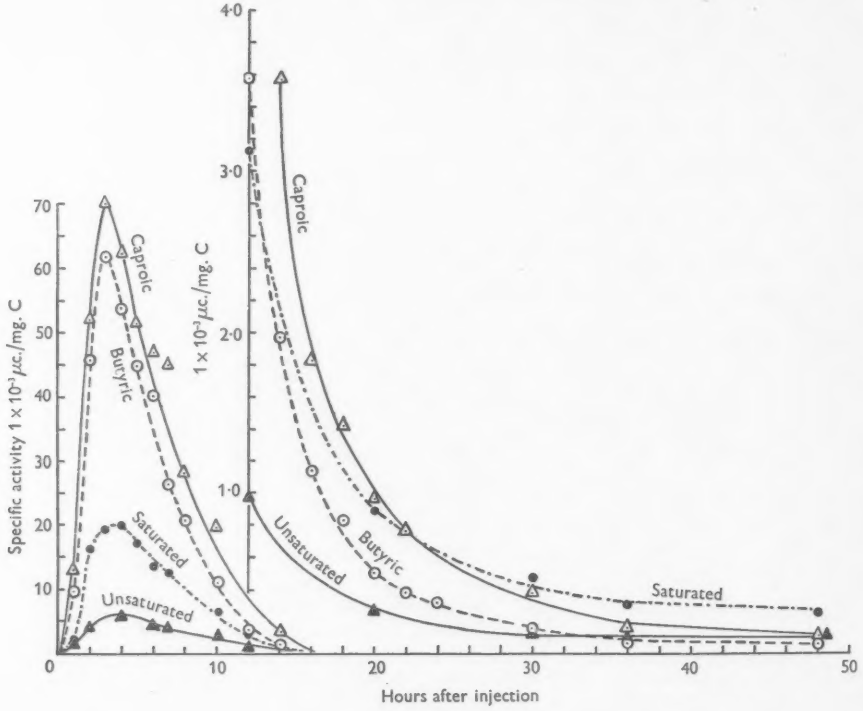

Fig. 1.

of it. The maximum specific activity of the plasma fatty acids was reached at about 24 hr. after the injection of the labelled acetate. At this time the milk fatty acids still contained more ^{14}C than the plasma fatty acids. If one were to determine the specific activity of plasma fatty acids 3–4 hr. after the injection by extrapolation from the specific activity-time curve, it would be found that the specific activity of all fatty acid fractions in the milk at this time was several hundred times that of the plasma fatty acids. Hence, it must be concluded that synthesis from small molecules in the udder is of outstanding importance in milk-fat formation. Further work is being planned to obtain individual fatty acids from our material and to carry out chemical degradation of these in order to obtain evidence as to the mechanism of fatty acid synthesis.

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Some Observations on the Adreno-cortical Steroids in Human Urine. By J. Y. F. Paterson, R. I. Cox and G. F. Marrian. (*Department of Biochemistry, University of Edinburgh*)

There are two general types of method for the estimation of urinary adrenal cortical steroids. One type of method estimates these steroids by the reducing power of suitably prepared extracts of urine (Talbot, Saltzman, Wixom & Wolfe, 1945; Heard & Sobel, 1946; Heard, Sobel & Venning, 1946). The other type of method estimates these steroids by the formaldehyde generated on oxidation of urine extracts with periodic acid (Lowenstein, Corcoran & Page, 1946; Daughaday, Jaffe & Williams, 1948). By either type of method the amount of adreno-cortical steroids which can be extracted from urine is increased by preliminary acidification of the urine. This suggests that these steroids may be excreted, at least in part, as conjugates.

In the present work the conditions necessary for the optimum hydrolysis of these presumed conjugates were studied, using a slightly simplified Daughaday method. Urine was acidified to pH 1, allowed to stand at 25°, and duplicate aliquots withdrawn for estimation at various time intervals. Estimations were also done on aliquots of the urine which had not been acidified. The hydrolysis curve so obtained was unexpected (Fig. 1, solid points), and indicates that the formaldehydogenic substances estimated by this method may be divided into two groups. The first group is acid labile, and the other stable to acid under the conditions used.

Heard *et al.* (1946) have observed that when urine is acidified, the amount of 'reducing substance' which can be extracted by chloroform-ether is increased. If, before extraction, the urine is neutralized immediately after acidification, the amount of reducing substance extracted is nearly the same as that extracted from unacidified urine. In the present work, hydrolysis curves, in which one of the duplicate aliquots was neutralized before extraction, were done. The results (see figure) confirm Heard's observations and indicate that both groups of formaldehydogenic substances are conjugated. The conjugates of the first (labile) group can be extracted by chloroform from acid urine, but not from neutral urine. The conjugates of the second (stable) group cannot be extracted by chloroform either from neutral or acid urine.

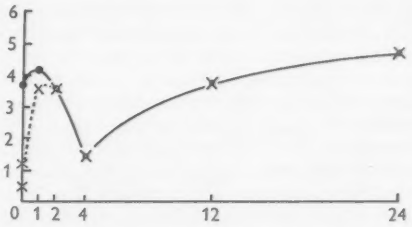

Fig. 1. Hydrolysis curve: normal male. Abscissa: time interval between acidification and extraction (solid points) or acidification and neutralization (followed immediately by extraction) (crosses) in hours. Ordinate: formaldehyde (μg.) per aliquot (1/15 of 24-hr. urine).

Increases in the amounts of both groups of substances have been found in pregnancy, in a case of Cushing's syndrome, and on administration of ACTH to a rheumatoid arthritic woman. Two abnormal hydrolysis curves have been observed.

The importance of the above results in routine estimations is stressed. Talbot uses extracts of unacidified urine; Heard *et al.*, Lowenstein *et al.* and Daughaday *et al.* extract immediately after acidification, while Mason & Sprague (1948) allow the urine to hydrolyse for 1–3 days. All of these are obviously not 'optimum hydrolysis conditions'.

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The Conversion of Carotene to Vitamin A in the Intestine of the Chick. By S. Y. Thompson, M. E. Coates and S. K. Kon. (*National Institute for Research in Dairying, University of Reading*)

It is now established that carotene is converted to vitamin A in the small intestine of the rat and of the pig (for references see Thompson, Ganguly & Kon, 1949). We have found that the same is true of the chick.

Day-old chicks received a diet of the following percentage composition, supplemented with 1000 B.S.I. units of vitamin D_3/kg.: weatings 25·5, ground wheat 20, ground oats 10, ground barley 10, casein 10, dried skim milk 6, groundnut meal 10, brewer's yeast 3, groundnut oil 2, $CaCO_3$ 3, NaCl 0·5. On this diet their vitamin A reserves were, as shown below, rapidly depleted and interference with growth became evident during the third week:

Age of chicks in days and number (in parentheses)	1 (2)	8 (2)	15 (1)	36 (4)	42 (1)
Vitamin A (i.u./liver)	11	22	2	1	2

The increase of liver reserves at 8 days was probably due to absorption of the vitamin from the yolk sac.

At 6 weeks some of the birds were starved overnight and then offered solid food for a few minutes before dosing by tube with 4 mg. β-carotene dissolved in 400 mg. groundnut oil. They were killed by decapitation 0·5, 1, 2 and 4 hr. after dosing. The small intestine was washed out immediately with saline. There was very little vitamin A in the contents. Fig. 1 shows that vitamin A, measured as described by Thompson *et al.* (1949), appeared in the intestinal wall within 0·5 hr., the shortest time interval studied, whereas the liver stores did not increase appreciably till 3·5 hr. later. For this reason chicks are likely to prove specially suitable for the study of the intestinal conversion of carotene. They are so easily rendered deficient that they may also be of value for the biological assay of vitamin A.

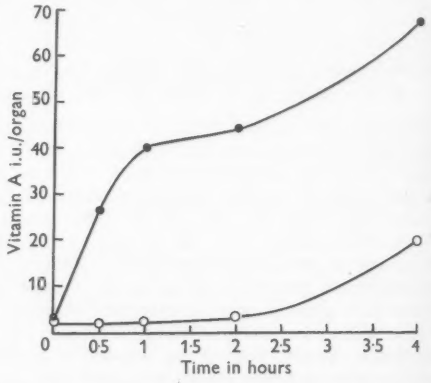

Fig. 1. Appearance of vitamin A in the small intestine (●—●) and liver (○—○) of vitamin A-deficient chicks after a meal of β-carotene. Mean values for two chicks.

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Conversion of Carotene to Vitamin A in the Intestine of the Pig and of the Rat: Transport of Vitamin A by the Lymph. By M. E. Coates, S. Y. Thompson and S. K. Kon. (*National Institute for Research in Dairying, University of Reading*)

In continuation of the work presented a year ago before this Society (Thompson, Braude, Cowie, Ganguly & Kon, 1949), plastic cannulas were placed in the mesenteric lymphatics of pigs and rats. For rats the technique was that of Bollman, Cain & Grindlay (1948), except that polythene, instead of polyvinyl chloride, tubing was used, and that the tube was pushed directly through the wall of the lymph vessel and not through a prepared hole. For pigs the procedure was essentially similar. After cannulation the rats were kept in the cage described by Bollman & Van Hooke (1948). Normal rats

were anaesthetized with Nembutal (Abbott Laboratories); those made vitamin A-deficient on the diet of Thompson, Ganguly & Kon (1949) were given ether.

In pigs, whether β-carotene (300 mg.) or vitamin A (100,000 i.u.) was given, the marked (100-fold) increase in the concentration of vitamin A in the lymph was almost exclusively in the ester form (cf. for carotene Thompson, Braude, Cowie, Ganguly & Kon, 1949; Thompson, Ganguly & Kon, 1949; for vitamin A, Drummond, Bell & Palmer, 1935; Eden & Sellers, 1948; Goodwin & Gregory, 1948).

In rats, whether normal or vitamin A-deficient, the increase was also mostly as ester. Vitamin A did not appear in the lymph in increased quantities for at least 1 hr. after the carotene meal; though conditions were not strictly comparable it is probably significant that we found it in the intestine within 15 min. of dosing (Thompson, Ganguly & Kon, 1949). In the vitamin A-deficient rat killed 2 hr. after the carotene meal appreciable quantities of vitamin A were present in the intestine and in the lymph collected between 1 and 2 hr. after the meal, but none appeared in the blood or in the liver. Yet in the uncannulated animal killed at the same time the increase in the blood and liver was marked (see Table).

Appearance of vitamin A in the small intestine, mesenteric lymph, blood and liver of vitamin A-deficient rats after a meal of about 4 mg. β-carotene in arachis oil. Animals dosed 3 hr. after cannulation of lymphatic

Treatment	Time after dosing (min.)	Small intestine total vitamin A (i.u./rat)	Lymph: Fluorescence in ultra-violet	Lymph: Yield (ml.)	Lymph: Vitamin A (i.u.) Alcohol	Lymph: Vitamin A (i.u.) Ester	Blood (i.u./rat) Alcohol	Blood (i.u./rat) Ester	Liver total vitaminA (i.u./rat)
Experiment 1									
Cannulated and dosed	0	—	Bluish	0·9	0·3	0·3	—	—	—
	30	—	Bluish	0·5	0·3	0·2	—	—	—
	60	—	Bluish	0·3	0·2	0·3	—	—	—
	75	12	Yellow	0·5	0·3	1·5	1·8	0·8	1·4
Not cannulated, arachis oil only	75	1·8	—	—	—	—	1·8	1·4	0·9
Experiment 2									
Cannulated and dosed	0	—	Bluish	2·0	0·2	0·2	—	—	—
	30	—	Bluish	0·5	0·2	0·1	—	—	—
	60	—	Bluish	0·7	0·2	0·1	—	—	—
	90	—	Yellow	0·7	0·4	4·0	—	—	—
	120	13	Yellow	0·8	0·4	8·6	1·4	0·9	2·1
Not cannulated, dosed	120	35	—	—	—	—	13·7	7·5	24·0
Not cannulated, arachis oil only	120	0·9	—	—	—	—	1·1	0·2	0·4

All these experiments, together with those reported earlier (Thompson, Braude, Cowie, Ganguly & Kon, 1949; Thompson, Ganguly & Kon, 1949), make it clear that the vitamin A arising from carotene in the intestine is carried thence by the lymph to the blood and by the blood to the liver.

We are greatly indebted to our colleagues: to Dr A. T. Cowie for the cannulation of the pigs, to Mr H. S. Hall for the special cages, to Dr K. M. Henry for the preparation of the rats and to Dr R. Braude for the preparation of the pigs.

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Ayfivin and Bacitracin. By G. G. F. Newton and E. P. Abraham. (*Sir William Dunn School of Pathology, University of Oxford*)

The antibiotic ayfivin, produced by a strain of *Bacillus licheniformis*, was partially purified by Sharp, Arriagada, Newton & Abraham (1949). The product was found to be a mixture of polypeptides and to resemble bacitracin. Arriagada, Florey, Jennings & Wallmark (1949) showed that crude ayfivin cured mice of certain systemic infections, but that it severely damaged the kidneys. Further experiments are now reported on the separation of the components of the crude product and on the relationship of ayfivin to bacitracin (Gregory, Barry & Craig, 1948, 1949).

Method. The method of counter-current distribution between solvents, developed by Craig and co-workers, was used in the present investigations. Most of the distributions were carried out in a solvent-system composed of amyl alcohol, *n*-butanol and phosphate buffer, pH 7 (system I), but one comparative experiment was made using a mixture of *sec.*-butanol and aqueous acetic acid (system II). The material in different fractions was estimated by hydrolysis and measurement of the colour density given by the resulting amino acids when treated with ninhydrin in the manner described by Moore & Stein (1948). Initial distributions of several grams of material were carried out in separating funnels. Selected fractions were then subjected to 100 transfers in an apparatus of the type described by Craig & Post (1949).

Results. By distribution in system I crude ayfivin was resolved, or partly resolved, into at least seven polypeptides, of which three (referred to as A, B and C) had antibacterial activity. Substance A was the major active constituent. Its specific activity against *Corynebacterium xerosis* was similar to that of C and about four times that of B. It appeared to be stable on redistribution in system I.

In contrast to its behaviour in system I, crude ayfivin showed very little resolution on counter-current distribution in system II.

The distribution curve of commercial bacitracin in system I resembled that of partially purified ayfivin, and a mixture of ayfivin A and the corresponding component of bacitracin showed no sign of resolution into two substances after 100 transfers. The main active constituents of ayfivin and bacitracin are certainly very similar, and possibly identical. It is proposed to abandon the name ayfivin and to call its active constituents bacitracin A, B and C.

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Some Observations of the Fate of *bis(dimethylamino)-phosphonous anhydride* in the Rabbit. By J. E. Gardiner and B. A. Kilby. (*Department of Biochemistry, School of Medicine, University of Leeds*)

A dose of 50 mg./kg. of *bis(dimethylamino)phosphonous anhydride*, $(Me_2N)_2PO.O.PO(NMe_2)_2$, injected into rabbits leads to death within a few hours with typical symptoms of acetylcholine poisoning (excessive salivation, fibrillary twitchings, etc.) as produced by fluorophosphonates and other anticholinesterases. However, when the action of this anhydride on cholinesterases is measured *in vitro*, surprisingly high concentrations are required to produce 50% inhibition, in contrast with other organic phosphorus compounds which are effective at concentrations of the order of 10^{-7} to 10^{-8} M:

Source of cholinesterase	Conc. of anhydride for 50% inhibition (M)
Human erythrocytes	$1{\cdot}1 \times 10^{-1}$
Whole rabbit blood	$2{\cdot}6 \times 10^{-2}$
Bee brain (Metcalf & March, 1949)	$> 1{\cdot}2 \times 10^{-3}$

We have investigated this apparent anomaly by a comparison of the effects of the anhydride on rabbit blood cholinesterase activity *in vivo* and *in vitro*. A sample of anhydride incorporating ^{32}P has been made (Gardiner & Kilby, 1949) and injected into rabbits (50 mg./kg. i.p.) and the blood cholinesterase

activity at death measured manometrically. In parallel, blood withdrawn before injection was incubated at 37° with known concentrations of anhydride. The apparent concentration of anhydride in the blood *in vivo* was followed by radioassay of 0·1 ml. samples taken at 5–10 min. intervals:

	Rabbit I	Rabbit II
Inhibition of cholinesterase at death (%)	67·7	95·4
Apparent concentration of anhydride in blood at death from ^{32}P assay (mg./ml.)	0·044	0·15
Concentration of anhydride required to produce same inhibition *in vitro* (mg./ml.)	17·5	166

The inhibitory action of the anhydride on rabbit-blood cholinesterase is enhanced by previous incubation with rabbit liver slices:

Inhibitor solution added to blood	Inhibition of esterase. Final conc. of anhydride $5{\cdot}5 \times 10^{-4}$M	$2{\cdot}8 \times 10^{-4}$M
Supernatant from liver slices incubated in buffer solution (3 hr. 35°) + buffer solution containing anhydride (%)	6·7	7·6
Supernatant from liver slices incubated in buffer containing anhydride + buffer solution (%)	36·5	29·5

These results can be explained by postulating the conversion of the anhydride *in vivo* into some more active inhibitory compound, the liver being one place where this can occur. The formation of the half molecule, $(NMe_2)_2PO(OH)$, is excluded as this is inactive.

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Nitrogenous Excretion of Amphipods and Isopods. By Elisabeth I. B. Dresel and Vivien Moyle (introduced by E. Baldwin). (*Biochemical Department, University of Cambridge, and Marine Biological Laboratory, Plymouth*)

There exists among vertebrates a marked correlation between the predominant end-product of nitrogen metabolism and the availability of water. Amphibious and terrestrial species are ureo- or uricotelic, whereas species enjoying an abundant water supply are ammonotelic. Similarly, among invertebrates, the insects, which are essentially terrestrial, are uricotelic.

A group of amphipod and isopod Crustacea taken from wholly marine, marine littoral, estuarine, freshwater and terrestrial habitats has now been studied. In all the species examined the predominant nitrogenous end-product was ammonia, which accounted for 50–90 % of the total non-protein nitrogen excreted. Urea and uric acid never accounted for more than 10 %.

That some form of metabolic adaptation has occurred in those species which are morphologically and physiologically adapted to terrestrial conditions is, however, apparent, for the total non-protein nitrogen output of the terrestrial species is only about one-tenth of that of the aquatic forms. Terrestrial adaptation in this group thus appears to be associated with an overall reduction of protein metabolism, a condition analogous to that which obtains in eggs of the cleidoic type.

Small amounts (5–10 %) of uric acid were found in the excreta of the terrestrial isopods and also of the fresh-water isopod, *Asellus aquaticus*. This uric acid might originate from the purines as a result of the loss of one or more uricolytic enzymes. It was accompanied by some retention of this insoluble compound, the amount so retained being correlated with the degree of adaptation to terrestrial conditions. An even greater retention, however, occurred in *A. aquaticus*.

Iodometric and Turbidimetric Determination of Mercapturic Acids in Urine. Mercapturic acid Excretion of Rabbits receiving Benzene. By D. V. PARKE and R. T. WILLIAMS. (*Department of Biochemistry, St Mary's Hospital Medical School, London, W. 2*)

Stekol (1936) estimated *p*-bromophenylmercapturic acid in rat and dog urine by hydrolysing the acid with 0·35N-NaOH to *p*-bromothiophenol which was determined iodometrically. In some cases the thiophenol was estimated gravimetrically as the mercuric mercaptide, but this method is inapplicable on a small scale. These methods, which are based on the original observations of Baumann (1882), are being investigated in detail.

Our studies have shown that mercapturic acids vary in their stability to alkali and are more stable than has been believed (see Table 1). Phenylcysteine is much more stable towards alkali than its *N*-acetyl derivative, phenylmercapturic acid, which is more stable than the halogen derivatives. In our estimations of mercapturic acids we used N-NaOH.

Table 1

Compound	Normality of boiling NaOH for complete hydrolysis in 30 min.
L-*p*-Chlorophenylmercapturic acid	0·4
L-*p*-Bromophenylmercapturic acid	0·4
L-Phenylmercapturic acid	0·75
L-*S*-Phenylcysteine	5·0

When mercapturic acids in urine are determined iodometrically, blank titrations are variable (cf. Binkley, 1949). In rabbits the blank variations can be reduced by careful selection of diets. However, in the phenylmercuric mercaptide method there is no blank, and it is more specific than the iodometric method. A turbidimetric procedure has been developed whereby mercapturic acid of the order of 1 mg. in 5 ml. urine can be estimated. In this method the opacity of suspensions of phenylmercuric mercaptide, produced in suitably treated urines by adding mercuric chloride, was estimated in the Spekker absorptiometer. Standard curves were made from suspensions of the mercuric mercaptide in 0·5N-Na_2SO_4 in 10% ethanol in water, stabilized with 0·01% gelatin. With urine gelatin is unnecessary.

With pure solutions of mercapturic acids, both methods give the same results, but in urine the iodometric method gives slightly higher results. Table 2 gives the mercapturic acid output in rabbits receiving benzene orally.

Table 2

Dose of benzene (g./kg.)	% dose excreted as mercapturic acid	
	Iodometric	Turbidimetric
1·0	1·6	1·3
1·0	0·8	0·65
1·0	1·2	0·8
1·0	0·75	0·6

Thus on the more specific turbidimetric method about 0·8% of the benzene appeared in the urine as a mercapturic acid (cf. Zbarsky & Young, 1943, for experiments on rats).

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A Colorimetric Assay Method for Vitamin B_{12}. By K. H. FANTES and D. M. IRELAND (*Research Division, Glaxo Laboratories, Ltd., Greenford, Middlesex*), and N. GREEN (*Research Division, Glaxo Laboratories Ltd., Barnard Castle, Co. Durham*)

A quantitative colorimetric method for assaying vitamin B_{12} in impure solutions could theoretically be based on (1) a change in the red colour of vitamin B_{12} to some different colour, or its specific decolorization, or (2) the colour of a hydrolytic product of vitamin B_{12} after preferential extraction to separate it from impurities.

Approach (1) was unsuccessful, although it was found that traces of chlorine in the presence of 5N-HCl would convert the red acid hydrolytic product of vitamin B_{12} to a substance with a blue-purple colour. The blue colour was formed by heating the vitamin B_{12} solution at 100° C. for 1 hr. with an equal volume of 10N-HCl, containing a trace of 16N-HNO_3 to generate chlorine. A slight excess of chlorine decolorized the solution, and the amount needed to produce the optimum blue colour was very critical. With impure preparations the amount of HNO_3 needed could not be predicted.

No oxidizing or reducing agents were found that specifically decolorized vitamin B_{12} or its acid hydrolytic product.

Lester Smith (1948) showed that the red hydrolytic product of vitamin B_{12}, an organic acid, was extracted by butanol. Ellis, Petrow & Snook (1949) mentioned the methyl ester of the acid. We have found that the red acid, on being shaken with a higher alcohol (e.g. octyl alcohol) in the presence of 5N-HCl, is slowly esterified, and that the ester so formed is quantitatively extracted into the alcohol. Addition of light petroleum does not transfer the ester to an aqueous or methanolic acid phase, but removes small amounts of coloured impurities.

The hydrolysis is done with an equal volume of 10N-HCl in sealed tubes at 100° for several hours. To convert the resultant organic acid to its ester it is necessary to shake the hydrolysate with an equal volume of *n*-octyl alcohol for 2 or 3 hr. An aliquot of the alcohol layer, diluted with up to seven volumes of light petroleum, is washed with a mixture of methanol:N-HCl (4:1). The colour of the octyl alcohol-petroleum solution is read in a 1 cm. or a 4 cm. micro-cell in a Hilger photoelectric absorptiometer with the use of an Ilford 604 filter.

Results were read from a standard curve. They were reproducible to $\pm 5\%$, and were in good agreement with those obtained by the microbiological method (Cuthbertson, 1949). Partly purified streptomyces fermentation liquors with a vitamin B_{12} content of 0·2% of the total solids and commercial liver extracts could be assayed by this method. The minimum amount of vitamin B_{12} that can be measured is about 40 μg. in not more than 2 ml. Related cobalt-containing factors of the vitamin B_{12} complex, such as vitamin $B_{12}b$ (Pierce, Page, Stokstad & Jukes, 1949), behave like vitamin B_{12} itself in this assay procedure.

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The Biogenesis of β-Carotene in the Fungus *Phycomyces blakesleeanus*. By G. A. Garton, T. W. Goodwin and W. Lijinsky. (*Department of Biochemistry, University of Liverpool*)

Phycomyces blakesleeanus is a very suitable organism with which to study carotenoid synthesis; it is easily cultured on liquid media and it produces only one carotenoid, β-carotene (Schopfer, 1935; Karrer & Krause-Voith, 1947). As little is known concerning carotenoid biogenesis, the first step was to determine quantitatively the production of carotene using the standard medium described by Schopfer (1934); this medium contains glucose (10%) as the C source and asparagine (0·2%) as the N source, together with KH_2PO_4 (0·15%), $MgSO_4.7H_2O$ (0·05%) and aneurin (25 μg. %). The cultures were incubated at 25° in a glass cabinet which received normal daylight illumination. The β-carotene was determined spectrophotometrically, and fat and dry-weight determinations were also made. The carotene content reached a maximum after 8–9 days and thereafter decreased quite rapidly; maximum dry weight and fat values were obtained after 3–4 days and thereafter remained constant. The results for the (+) and (−) strains of the fungus followed the same pattern, but the (+) strain produced only about half as much β-carotene as did the (−) strain, although the fat and dry weight production was of the same order. In further investigations only the (−) strain is being used.

When *Phycomyces* was cultured on the standard medium in the dark, β-carotene production was only about one-half of that produced in the light, whilst the fat and dry weight were not affected. These observations are contrary to those of Schopfer (1943) who reported no β-carotene formation in the absence of light.

When asparagine was replaced by L-valine, L-alanine or L-leucine no appreciable differences in carotene, fat or dry weight production could be detected, but replacement by glycine resulted in a marked and specific stimulation of carotenogenesis; L-*iso*leucine did not produce appreciable growth and NH_4NO_3 produced results similar to those obtained using asparagine until the pH of the medium fell below 3·4, when growth ceased.

Carotenogenesis appears to depend on the metabolism of exogenous nitrogen. If the culture medium is removed from a 3-day-old mycelium and replaced by a medium containing glucose but no nitrogen, fat and carbohydrate production proceeds normally, whereas β-carotene formation ceases.

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Chromatography of 2:4-Dinitrophenylhydrazones of Ketoacids on Alumina. By S. P. Datta, H. Harris and K. R. Rees. (*Department of Biochemistry and Galton Laboratory, University College, London*)

This method has been developed to investigate quantitatively the ketoacids in urine and other body fluids.

Alumina, 200 g. (Peter Spence Ltd., Type 'H'), is packed in ethyl acetate in a column 2·5 cm. in diameter. The mixture of 2:4-dinitrophenylhydrazones is applied to the column in 75 ml. ethyl acetate. The chromatogram is developed with ethyl acetate, ethanol, aqueous ethanol and dilute alcoholic sodium carbonate solution in that order. A pressure of 25 cm. Hg of CO_2 is used to speed up the rate of flow of the solvents. 15 ml. fractions are collected from the column and their yellow colour measured in a photoelectric absorptiometer using a 1 cm. cell and a Chance OB1 blue light filter.

according to the particular 2:4-dinitrophenylhydrazones it is desired to investigate and the relative amounts present. Thus to effect a complete separation of the 2:4-dinitrophenylhydrazones of phenylpyruvic and *p*-hydroxyphenylpyruvic acids the amount of 95% ethanol passed through the column is increased; similarly if the separation of the dibasic ketoacid 2:4-dinitrophenylhydrazones is desired a less alkaline eluant (0·1 M-Na_2CO_3) may be used with advantage.

In applying the method to urine the following procedure is adopted. The urine is treated with an equal volume of 0·01 M 2:4-dinitrophenylhydrazine in 2 N-HCl and allowed to stand at room temperature for 24 hr. The 2:4-dinitrophenylhydrazones thus

Table 1

Solvent mixtures

	(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)
Ethyl acetate	1	1	1	–	–	–	–	–
95% ethanol	–	1	3	1	9	1	1	1
Water	–	–	–	–	1	1	3	–
0·5 M-Na_2CO_3	–	–	–	–	–	–	–	3
Total volume used in ml.	850	400	200	400	1000	400	300	600

Movement of 2:4-dinitrophenylhydrazones

	(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)
Acetone	+ + +	·	·	·	·	·	·	·
Acetaldehyde	+ + +	·	·	·	·	·	·	·
Benzaldehyde	+ + +	·	·	·	·	·	·	·
Phenylacetaldehyde	+ + +	·	·	·	·	·	·	·
Phenylpyruvic acid	–	–	–	+	+ + +	·	·	·
p-Hydroxyphenylpyruvic acid	–	–	–	±	+ +	·	·	·
Pyruvic acid	–	–	–	–	+	+ +	·	·
Oxalacetic acid	–	–	–	–	–	–	–	+ +
α-Ketoglutaric acid	–	–	–	–	–	–	–	+

The table indicates the solvent mixtures and volumes we have found useful and also the movements of the various 2:4-dinitrophenylhydrazones we have investigated.

It will be seen from the table that the neutral 2:4-dinitrophenylhydrazones are eluted immediately by ethyl acetate and do not interfere with the subsequent separation of those of the keto-acids.

Using suitable calibration curves made from standard solutions of the various 2:4-dinitrophenylhydrazones in the solvent in which they are eluted from the column, we have been able to estimate quantities of ketoacids of the order of 1 mg. with an error of less than 5%.

The volumes of the various eluants may be varied

formed may be extracted with ethyl acetate and applied to the column *in toto*, or may first be fractionated by suitable partition between ethyl acetate and M-Na_2CO_3 solution into neutral and acidic fractions. The final solution in ethyl acetate should be dried over Na_2SO_4 before being applied to the column. A suitable amount of urine for the size of column described is of the order of 1/10th of a 24 hr. specimen in the case of normal subjects and down to 1/200th of a 24 hr. specimen in certain pathological conditions.

Several different ketoacids have been found to occur regularly in normal human urine, these are being identified. The method is also being applied to the study of urine from cases of phenylketonuria.

Production and Utilization of Methylglyoxal by Tissue. By H. M. Salem and E. M. Crook. (*Department of Biochemistry, University College, London*)

Dakin & Dudley (1913) discovered enzyme glyoxalase which catalyses the reversible conversion of methylglyoxal into D-lactic acid in all animal tissues. They also found an inhibitor antiglyoxalase in the pancreas. Toenneissen & Fisher (1926) showed that hexose-diphosphate was converted to methylglyoxal when the latter was digested with muscles in the presence of antiglyoxalase to retard the action of glyoxalase on methylglyoxal.

Ariyama (1928) repeated these experiments but failed to show the influence of antiglyoxalase.

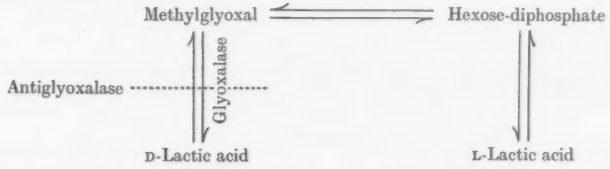
It has now been confirmed that when hexose-diphosphate is incubated with liver homogenate, methylglyoxal is produced. The methyl glyoxal formed was measured quantitatively by a method using glyoxalase.

Ariyama's suggestion that the enzyme glyoxalase was destroyed by incubation was disproved, and evidence was obtained to show that it was the glyoxalase coenzyme, glutathione, which was destroyed, thus resulting in the inactivation of glyoxalase. Evidence was also obtained to show that the reaction

Hexose-diphosphate ⇋ methylglyoxal

was reversible. When methylglyoxal was incubated with liver homogenate and antiglyoxalase which inhibited the destruction of methylglyoxal by glyoxalase, half of the total inorganic phosphorus and three quarters of the methylglyoxal disappeared. The disappearing inorganic phosphorus was found to accumulate in the hexose diphosphate and phosphopyruvic fractions. The latter might be derived from the first through glycolysis, and could itself give rise to lactic acid by further breakdown.

The following scheme would summarize the whole system:



The observation by Dakin & Dudley (1913) that a mixture of both D- and L-lactic acid was formed in unequal proportions when methylglyoxal was incubated with crude glyoxalase preparations could then be explained. Glyoxalase acting on methylglyoxal produced D-lactic acid. At the same time, some methylglyoxal was converted to hexose-diphosphate by the reversible reaction shown above and the latter was converted to L-lactic acid through glycolysis.

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The Mode of Action of Glyoxalase. By E. M. Crook and Kathleen Law. (*Department of Biochemistry, University College, London*)

Glyoxalase, in the presence of reduced glutathione ('GSH'), converts substituted glyoxals to the corresponding hydroxy acids.

$$R.CO.CHO \xrightarrow[H_2O]{} R.CHOH.COOH$$

(Dakin & Dudley, 1913).

By iodine titration of the —SH groups, Jowett & Quastel (1933) obtained evidence for the formation of a methylglyoxal —GSH compound in the absence of enzyme. Giršavičius (1935) showed that in the presence of glyoxalase there was a similar disappearance of free —SH groups, indicating the formation of some type of methylglyoxal —GSH complex.

The scheme of reaction postulated at that time was as follows:

$$CH_3.CO.CHO + GSH \rightleftharpoons CH_3.CO.CHOH.SG \xrightarrow[H_2O]{} CH_3.CHOH.COOH + GSH.$$

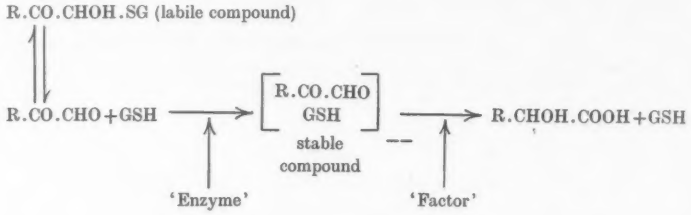
In 1936 Yamazoye showed that the compound formed from methylglyoxal and GSH in the presence of crude enzyme was different from that produced

when the substrates react in the absence of enzyme. The latter was labile, readily regenerating methylglyoxal and GSH; its —SH groups reacted when an excess of I_2 was added and it gave strong reactions for keto-groups. The compound formed in the presence of the enzyme was much more stable; it did not regenerate methylglyoxal and GSH but was slowly hydrolysed above pH 7 to GSH and lactic acid, and gave no reactions for keto-groups.

Hopkins & Morgan (1948) separated and purified two components of glyoxalase. They showed that the 'enzyme' acts on methylglyoxal and on phenylglyoxal at the same rate. The second component or 'factor' accelerates the conversion of methylglyoxal to lactic acid, but causes no increase in rate when phenylglyoxal is the substrate.

In this communication evidence will be given which shows that the formation of the stable glyoxal —GSH compound is brought about by the 'enzyme'. This enzymic compound under experimental conditions breaks down spontaneously into the corresponding hydroxy acid and GSH. The 'factor' accelerates the breakdown of the methylglyoxal —GSH compound, but has no action on the phenylglyoxal compound.

The reactions are shown to be:



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The Mechanism of the Anti-Aneurin Activity of Bracken (*Pteris aquilina*). By W. C. Evans, N. R. Jones and R. A. Evans. (*Biochemical Laboratories, Department of Agriculture, University College of Wales, Aberystwyth*)

It is now established that the inclusion of air-dried green bracken in the feed of rats (Weswig, Freed & Haag, 1946; Evans & Evans, 1949; Thomas & Walker, 1949) and horses (Roberts, Evans & Evans, 1949) produces an avitaminosis B_1 in these animals, although the diet as a whole is originally adequate in its aneurin content. In bovine bracken poisoning, evidence is accumulating (Hughes & Evans, unpublished) showing that when clinical symptoms are evident, there is also a state of B_1 deficiency, although this condition in the ruminant appears to be complicated by other factors.

The 'anti-aneurin factor' of bracken has now been shown to possess the following properties:

(1) It is very stable in the intact leaf after air drying at room temperature, and withstands heating at 100° C. for a considerable time in this condition. Steaming or autoclaving results in a rapid inactivation.

(2) De-proteinized water extracts, and ethanol concentrates of bracken, contain no demonstrable anti-aneurin activity.

(3) The factor is extracted from the leaf by chloroform—water at pH 7·5–8; 10 g. green leaf powder extracted in this way with 50 ml. solution overnight at 0° C. and filtered, gives a clear extract able to inactivate 5–10 μg. aneurin per ml. The anti-aneurin factor in this solution is thermolabile, and behaves like an enzyme. It can, however, be freeze-dried without loss of activity.

(4) By the use of *Neurospora crassa* mutant 9185, and *Phycomyces blakesleeanus* as indicators, it has been made highly probable that cleavage of the aneurin molecule occurs at the methylene bridge by the action of the bracken enzyme.

(5) This view of the chemical course of aneurin inactivation is supported by chromatographic evidence; the presence of the thiazole component as one of the products of reaction has been definitely established by this technique. Chemical isolation is in progress.

(6) A low temperature concentrate of the bracken enzyme (activity 100 μg. B_1/ml.) when added to a standard basal diet adequate for the growth of rats

has the same effect on the animals as the original bracken leaf powder diet, whilst a parallel experiment with a boiled concentrate added to the diet is quite innocuous and allows normal growth.

The existence of a plant thiaminase has not, as far as we are aware, been reported on before; there are indications that bracken is not the only plant possessing it, *Equisetum arvense* (horse-tail) for instance has been shown by us to contain it.

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The Avidity of Amino-acids for the Ions of Heavy Metals. I. Amino-acids with only two Ionizing Groups. By A. ALBERT. (*Department of Medical Chemistry, The Australian National University*)

It has long been known that various amino-acids form sparingly ionized complexes with the ions of heavy metals. So far, quantitative data has been available only for glycine (Flood & Loras, 1945). The discovery that pteridines (including folic acid) form similar complexes (Albert & Brown, 1949) made it desirable to obtain quantitative data for the amino-acids which would be important natural competitors. The discovery of comparable chelating properties in riboflavine and certain purines (Albert, unpublished) adds point to the investigation.

The following are the logarithms of the stability constants for the following equilibrium:

$$\frac{[\text{complex}]}{[\text{metal ion}]\,[\text{free anion}]^2}$$

(determined potentiometrically in 0·01 M aqueous solutions at 20°).

It is evident that each of those amino-acids that were tested with all eight metals has the same order of preference for metallic ions, copper always being the most firmly bound. There is no reason to expect any other amino-acid to depart from this order, provided it has only two ionizing groups. Proline complexes have the highest stability and asparagine complexes are among those that have the least. Nevertheless, at pH 7·3 asparagine can out-compete proline for securing metallic ions. This is because of the low basic pK_a of asparagine which ensures a high concentration of anions and hence (by the mass action law) a greater quantity of complex is formed. Hence the function $\bar{n}$ (Bjerrum, 1941), which represents the fraction (of the theoretically formable amount of complex) actually formed at a given pH, is of greater interest to biochemists than the stability constant, from which it can be derived.

	Cu^{++}	Ni^{++}	Zn^{++}	Co^{++}	Cd^{++}	Fe^{++}	Mn^{++}	Mg^{++}
Glycine	15·4	11·0	9·3	8·9	8·1	7·8	5·5	*c.* 4
Alanine	15·1	—	—	8·4	—	7·3	—	—
Valine	15·1	—	—	8·6	—	6·8	—	—
Phenylalanine	14·9	—	—	7·9	—	6·3	—	—
Serine	14·6	—	—	8·0	—	7·0	—	—
Methionine	14·7	—	—	7·9	—	6·7	—	—
Proline	16·8	11·3	10·2	9·3	8·7	8·3	*c.* 5·5	<4
Tryptophan	15·9	10·2	9·3	8·5	8·1	7·6	*c.* 5	<4
Asparagine	14·9	10·6	8·7	8·4	6·8	6·5	*c.* 4·5	*c.* 4

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Chemical Changes in the Developing Chick Embryo Related to the Deoxyribonucleic Acid Content of the Nucleus. By J. N. DAVIDSON, I. LESLIE, R. M. S. SMELLIE and R. Y. THOMSON. (*Biochemistry Department, The University, Glasgow*)

Deoxyribonucleic acid (DNA) has been found to be constant in amount in nuclei isolated from cells of different tissues of animals of a single species (Vendrely & Vendrely, 1948, 1949; Mirsky & Ris, 1949). Recently Davidson & Leslie (1950) have shown the value of this constant element of the cells in revealing chemical changes occurring in growing tissues. The composition of the predominant cell type is obtained when components of the tissues are expressed as amounts per unit of DNA. In this way it is possible to avoid some obscuring influences of variable tissue composition on results expressed in terms of wet or dry weights.

(2) Prior to hatching PN per cell decreases in all four tissues. The cell content of PN rises sharply to nearly twice its embryonic level 2–3 days after hatching.

(3) Brain is characterized by the relatively large increase of LP and RNAP per cell throughout embryonic growth and after hatching. Only in embryonic heart cells do LP and RNAP remain constant or decrease slightly during the period investigated.

These rising amounts of cellular components in brain, liver, and muscle are believed to reflect an aspect of the differentiation of these tissues during their development (Davidson & Leslie, 1950).

Table 1. *The deoxyribonucleic acid content of the nuclei of cells of different tissues of the fowl expressed as* $\mu g. \times 10^{-7}$ *deoxyribonucleic acid phosphorus (DNAP) per nucleus*

DNAP	Erythrocyte	Liver	Kidney	Spleen	Heart	Pancreas
By phosphorus determination	2·49	2·56	2·20	2·54	2·45	2·61
By diphenylamine test	2·43	2·33	2·23	2·40	2·45	2·32
By ultra-violet absorption	2·49	2·52	2·37	2·44	2·53	2·60

Table 2. *Growth rates* ($k = \tan \alpha$) *relative to growth rate of DNAP per organ*

Organ	Incubation period (days)	Acid-soluble phosphorus	LP	RNAP	PN
Brain: (1)	8–14	2·07	2·28	1·95	2·04
(2)	15–20	1·28	1·61	1·27	1·31
Heart	8–20	0·99	0·99	0·98	1·04
Liver: (1)	8–14	1·09	1·26	1·07	1·23
(2)	15–20	1·06	1·02	0·94	1·06

Preliminary results (Table 1) have confirmed that the amount of DNA is constant per nucleus in cells from a number of different organs of the adult fowl. When the protein nitrogen (PN), lipid phosphorus (LP), and ribonucleic acid phosphorus (RNAP) of the embryonic chick brain, heart, liver, and skeletal muscle are expressed as amounts per unit of deoxyribonucleic acid phosphorus (DNAP), i.e. as amounts per cell, and are plotted against incubation age, the following information is obtained:

(1) In brain, liver and skeletal muscle, PN increases per cell from the 8th to 18th day of embryonic development. In heart it remains more or less constant.

The growth rates of the various components have been compared with the growth rate of DNAP per organ in brain, heart, and liver (cf. Teissier, 1931; Needham, 1934). These are shown in Table 2 where the results are based on a larger number of determinations than those published previously (Davidson & Leslie, 1950). In brain the relative growth rates for PN, LP, and RNAP are characteristically high, suggesting that growth in this tissue involves a greater increase in cell size than occurs in other tissues. The discontinuities in the relative growth rates of components of brain and liver at 14 to 15 days confirm earlier observations of a critical phase in embryonic development at this stage (Needham, 1931).

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The Thermodynamics of the Active State of Muscle. By A. V. Hill. (*Biophysics Research Unit, University College, London*)

If a quick stretch is applied to a muscle shortly after a single shock the resistance to stretch is found to rise rapidly from the middle of the latent period onwards, i.e. before any shortening is detected in an ordinary contraction. The state of enhanced rigidity made evident in this way begins at about the same moment as the heat production and as the increase of optical transparency described by D. K. Hill (1949). It reaches its full extent very early, soon after the latent period is over; it is maintained for a time and then gradually disappears in 'relaxation'.

Stretched during contraction a muscle resists strongly, with a force substantially greater than that developed isometrically. The force diminishes sharply directly the stretch ends. The work (W) done in stretching it and the heat (H) produced are measured. If the stretch is timed to occur during the earlier phase of contraction (as distinguished from relaxation) ($H-W$) is considerably less than the heat which the muscle would have produced had it not been stretched. The stretch is applied between lengths at which the tension at rest is negligible, so that none of the work remains as elastic potential energy at the time when the total heat is measured after relaxation. A substantial part, therefore, of the work is absorbed. If the stretch is applied later, so as to fall partly during the phase of relaxation, the absorption of work is less. If applied wholly during relaxation, all the work appears as extra heat. The power of absorbing work, like that of doing work, is clearly a property of the active state. If activity is maintained by repeated stimuli the whole of the work done in stretching a muscle can be absorbed.

It is difficult not to suppose that the work which disappears is used in driving some chemical process backwards, presumably the same process, reversed, as supplies work when a muscle normally shortens. There is positive heat production associated with shortening as such and negative heat of lengthening. In the equation, therefore (A. V. Hill, 1949),

$$E = A + ax + \int P dx$$

(energy = activation heat + shortening heat + work),

the sign of x may be either positive or negative. The active state appears to involve a physical framework mechanically coupled with reversible chemical reactions.

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Volatile Fatty Acids in *Ascaris lumbricoides*. By E. Baldwin and Vivien Moyle. (*Biochemical Department, University of Cambridge*)

DEMONSTRATIONS

Fractionation of Hydrolysis Products of Amylose by Electrokinetic Ultrafiltration in an Agar-agar Jelly. By R. L. M. Synge (*The Rowett Research Institute, Bucksburn, Aberdeenshire*) and A. Tiselius (*Institute of Biochemistry, Uppsala*)

It should be possible to effect separations depending on differences in the rates of ultrafiltration of solutes by using an indefinitely extended ultrafilter such as a jelly. Experiments using pressure to force solvent through jellies resulted in their mechanical breakdown. It was therefore proposed to use the electrokinetic flow due to a potential gradient; here, frictional forces due to flow are throughout the gel opposed by electrically produced forces. The authors and Dr A. J. P. Martin have frequently considered these possibilities (cf. *Discussions Faraday Soc.* no. 7 (1949) 'Chromatographic Analysis').

The products of hydrolysis of amylose seemed promising test substances, since they are (Swanson & Cori, 1948) a randomly produced mixture of chain molecules, whose iodine colours are correlated with chain length (Swanson, 1948). An amylose fraction from potato starch (Bourne, Donnison, Peat & Whelan, 1949) was hydrolysed with 10N-HCl for 20 min. at 20°; the HCl was evaporated *in vacuo* and the neutralized product was incorporated as inlay in the apparatus of Consden, Gordon & Martin (1946, method B) using final concentrations: sodium acetate 0·02N; acetic acid 0·02N; iodine (dissolved with KI) 0·001N; agar-agar (cf. Gordon, Keil & Šebesta, 1949) 1% (w/v); hydrolysed amylose (inlay only) 0·2% (w/v). With 4 V./cm. separation of differently coloured components of the mixture was observed,

yellow migrating fastest, followed by orange, red, pink, lavender and blue, all with the electroendosmotic stream towards the cathode. Some blue material, perhaps phosphorylated, migrated anodically. Without hydrolysis of the amylose no migration or diffusion whatever occurred. Prolonging the hydrolysis diminished the slower blue-pink components relative to those staining red-orange.

The hydrolysed amylose was also investigated in the standard electrophoresis apparatus, in absence of agar, using the same buffer medium. Even after prolonged electrophoresis (1072 min. at 2·87 V./cm., +0·6° C.) only two boundaries were observed. The main component showed no mobility and no sign of inhomogeneity. The second component moved quite fast towards the anode (mobility 17·4 cm.^{2} sec.$^{-1}$ V.$^{-1}$) and is presumably identical with the anodic material of the agar experiment.

The separation in agar could reasonably be attributed to a molecular-sieve mechanism. The considerable sensitivity to agar concentration of the relative rates of the components is suggestive of this. With adsorption also occurring and following Traube's Rule, separation will be assisted. In ordinary, grossly particulate chromatograms, molecular-sieve action within the granules counteracts the operation of Traube's Rule. Electrokinetic ultrafiltration may therefore prove generally useful for separating substances chemically similar but differing in molecular weight.

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Apparatus for Mass-Production Two-Way Paper Chromatography. By S. P. Datta, C. E. Dent and H. Harris. (*Department of Biochemistry, University College, London, and University College Hospital, London*)

The principle involved here enables large numbers of two-way (two-dimensional) chromatograms to be run simultaneously. An adaptation for 12 paper squares 20 × 20 cm. is shown in Fig. 1 and in the demonstration. The solution to be analysed is placed near the corner of the paper as usual, and the papers threaded on the four rods of the frame. Collars of 2 cm. length are also threaded between the papers so as to keep them from touching each other. The frame and papers are placed in a dish containing the first solvent and the whole enclosed in any suitable box. The solvent soaks upwards until it reaches the far edge. The papers, still held on the frame, are dried, and then run with their other edge downward in the second solvent. They are again dried in the frame.

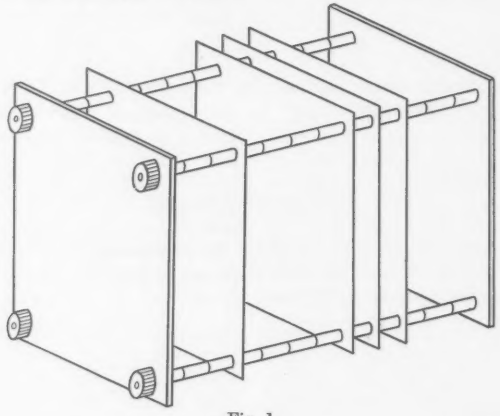

Fig. 1.

We find the apparatus valuable for routine urine testing. The resolving power for the amino acid spots is not quite as good as with the larger chromatograms (18 × 22 in. or 30 × 30 in.). The frame is made of duralumin. It has been found to be satisfactory for use with phenol and collidine-lutidine as solvents.

The Thermodynamics of the Active State of Muscle. Equipment and Methods. By A. V. Hill. (*Biophysics Research Unit, University College, London*)

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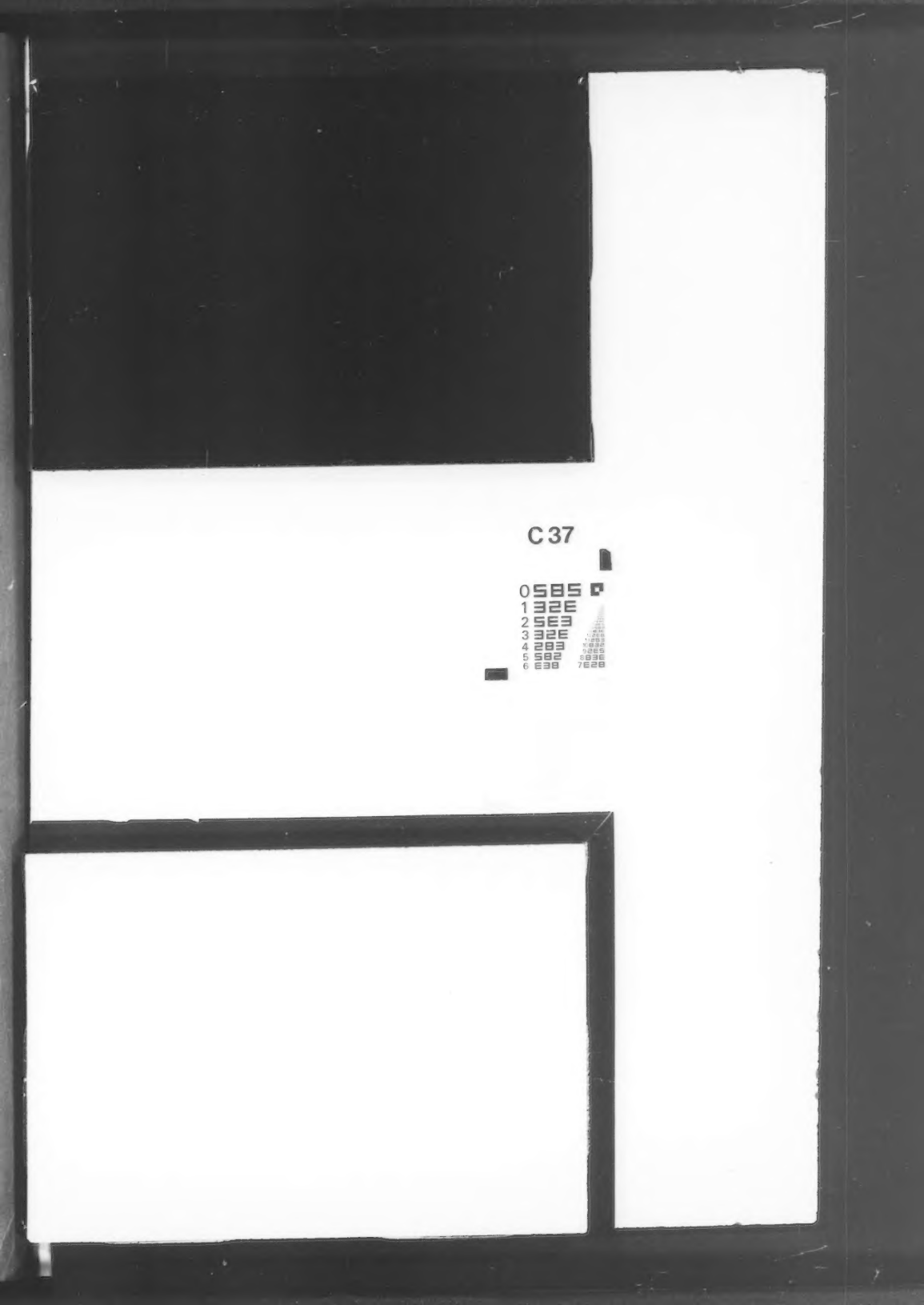
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